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The effects of anoxia and temperature on the development and survivorship of *Hexagenia* (Ephemeroptera: Ephemeridae) embryos, and implications for western Lake Erie populations

by

Jocelyn Gerlofsma

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Abstract

Benthic *Hexagenia* embryos can be exposed to anoxic hypolimnetic water or can become buried in the sediment. In western Lake Erie, periodic anoxia resulting from transient stratification has eradicated *Hexagenia* populations. The ability of embryos to survive anoxia and hatch when normoxic conditions return would allow the population to recover after an anoxic event. If the embryos can survive burial, the resurfacing of eggs after storms could account for the multiple cohorts typical of many populations. In the laboratory, I studied the survivorship and development of *Hexagenia* embryos at various developmental stages maintained in anoxic conditions for different time periods, at different temperatures.

Survivorship decreased log-linearly with term of exposure to anoxic conditions. After 245 d in anoxia at 20°C, viability was 16 percent. Temperatures < 20 °C did not influence viability under anoxic conditions. Stage of embryonic development had only a minor effect on ability to withstand anoxia.

Development was arrested by exposure to anoxia; time to hatching was delayed by the amount of time the eggs were in anoxia, regardless of the stage of embryonic development.

In 1996, *Hexagenia* nymphs were absent from several regions in western Lake Erie. I collected sediment cores from two such areas and two other areas supporting nymphs. Apparently viable eggs occurred in all cores. I studied the effects of hypoxia on embryos by adding eggs to the sediment cores. Hypoxia significantly reduced the

percentage of embryos hatching. Noteworthy observations of nymph burying eggs into the sediment were made in cores containing large nymphs.

The results of my studies are consistent with my thesis that *Hexagenia* eggs would be able to survive periods of anoxia at different temperatures over its embryological development. This would allow eggs to repopulate an area after a period of epibenthic anoxia that would eradicate the nymphs. It could also allow for the formation of an eggbank in the sediment.

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GENERAL INTRODUCTION

I examined the effects of anoxia on the development time and the hatching success of *Hexagenia* mayfly embryos. The effects of anoxia over a range of temperatures (Chapter One) and at different embryological developmental stages (Chapter Two) were investigated. I also examined the effects of hypoxia on the embryos in sediment from western Lake Erie (Chapter Three). Below, I illustrate why the effects of anoxia can be important to the *Hexagenia* nymph population, especially in a system like the western basin of Lake Erie.

The organism

The burrowing mayfly, *Hexagenia*, widespread throughout North America (Edmunds *et al.* 1976), is an important benthic invertebrate. *Hexagenia* mayflies are important as part of the aquatic food chain, as a major food source for many economically important fish (Neave 1932, Needham *et al.* 1935, Hunt 1953). The adults are considered a nuisance species. During periods of mass emergence, the adults collect under lights, creating odorous piles that can interfere with automobile traffic by creating slippery conditions (Hunt 1953). Another significant factor of *Hexagenia*, is their value as water quality indicators. They are used in toxicity testing (Freisen 1979, Ciborowski *et al.* 1991, Ciborowski and Corkum 1988, Kovats and Ciborowski 1989) and as biological monitors (Fremling 1989, Fremling and Johnson 1990, Reynoldson *et al.* 1989).

Hexagenia has a one to four year life cycle (Hunt 1953, Heise *et al.* 1987, Giberson and Rosenberg 1992a). It spends most of its life cycle as a nymph burrowed in the benthic

sediments of large rivers and lakes (Hunt 1953). From the nymph stage, it emerges as an immature winged stage called the subimago. Emergence occurs between mid-June and mid-August. The subimago stage lasts one to two days in the vegetation along the shoreline before molting to the mature imago stage (Hunt 1953). The imagos form mating swarms at dusk, along the shoreline edge. Once the eggs are fertilized the female oviposits them on the water's surface, and then dies (Hunt 1953). The eggs sink to the bottom, landing on the sediment surface, or aquatic vegetation, where they will hatch to the nymph stage.

Hexagenia nymphs have a low tolerance of pollution and low oxygen levels, as demonstrated by the loss of nymph populations in many North American lakes and large rivers between the 1930s and 1960s with increased pollution and eutrophication leading to epibenthic anoxic conditions (Britt 1955a, b, Jacobsen 1966). *Hexagenia* nymph populations were eliminated from Oneida Lake, New York (Jacobsen 1966), the Mississippi River (Fremling 1973), Green Bay of Lake Michigan (Howmiller and Beeton 1971), Saginaw Bay, Lake Huron (Schneider *et al.* 1969) and western Lake Erie (Carr and Hiltunen 1965). Improvements in water quality, resulting from the establishment of pollution abatement programs in the 1970s, have allowed for the return of *Hexagenia* in the upper Mississippi River (Fremling and Johnson 1990), in the lower Fox River draining into Green Bay, Lake Michigan (Cochran 1992) and western Lake Erie (Krieger *et al.* 1996).

Hexagenia in western Lake Erie

Hexagenia mayflies were dominant in the western basin of Lake Erie (Wright 1955, Chandler 1963), until the early 1950s, when the population drastically declined and then

disappeared in the 1960s throughout the basin (Carr and Hiltunen 1965, and Britt *et al.* 1973). There was a shift in the benthic community from a *Hexagenia-Oecetis* (caddisfly) community (Shelford and Boesel 1942) to one dominated by oligochaetes and chironomids (Beeton 1961, Carr and Hiltunen 1965).

The initial decline in the *Hexagenia* population was caused by a major anoxic event in 1953 (Britt 1955a). Anoxic conditions were produced during an extended period of stratification that allowed oxygen to become depleted in the hypolimnion (Britt 1955a).

The nymph population recovered the following summer in 1954 (Britt 1955b), but then fell again. The decline in the *Hexagenia* population was attributed to the eutrophication and increased pollution in the western basin (Britt 1955a, Carr and Hiltunen 1965, Reynoldson *et al.* 1989). The main effect of the eutrophication is thought to be an increase in the frequency of anoxic events that were recorded over that time in the basin (Britt 1955a, Carr *et al.* 1965, Britt 1968, Bartish 1984).

It was not until the early 1990s that *Hexagenia* started reappearing in western Lake Erie (Krieger *et al.* 1996, Schloesser *et al.* in review). The recovery was not uniform throughout the basin. In 1982, nymphs were found only at the mouths of the Detroit and Maumee rivers, along the west end of the western basin (Krieger *et al.* 1996). In the early 1990s, the distribution of nymphs gradually expanded eastward into the rest of the basin, presumably extending from those persistent populations at the mouth of the Detroit River (Krieger *et al.* 1996). By 1997, nymphs could be found throughout the basin except for a few areas, one being in the middle of Pigeon Bay and another south of Middle Sister Island (Ciborowski *et al.* 1998, and pers. observ.). The continued absence of nymphs in these areas

could be due to persistence of transient anoxic conditions. The reappearance of *Hexagenia* nymphs in the basin is attributed to water quality improvements resulting from pollution abatement programs and possibly from the establishment of the introduced zebra mussel, *Dreissena polymorpha* (Reynoldson *et al.* 1989, Kreiger *et al.* 1996, Schloesser *et al.*, in review).

Anoxia in western Lake Erie

The western basin is one of three basins in Lake Erie. The other two are the central, and the eastern basins. The western basin is the smallest, and shallowest of the three. It has a mean depth of 7.4 m and maximum depth of 18.9 m (Bolsenga and Herdendorf 1993). It is separated from the central basin on the west side by Point Pelee and a series of islands extending south of the point.

Anoxia in western Lake Erie occurs due to a depletion of epibenthic oxygen during transient periods of thermal stratification (Britt 1955a, 1963, Carr *et al.* 1965, Bartish 1984). Stratification is the layering of the lake water into the top, warm, circulating waters of the epilimnion and into the bottom, cold, relatively undisturbed waters of the hypolimnion (Wetzel 1975). The epilimnion and hypolimnion are separated by the thermocline, a region of a steep thermal gradient. The western basin, being relatively shallow and having a long wind fetch (approximately 60 km), usually allows the basin to remain well mixed (isothermal), but intermittent periods of stratification can occasionally develop during periods of calm weather (Chandler 1940, 1944, Wright 1955, Bartish 1984).

In the western basin, stratification was characterized by Bartish (1984) with the

thermocline occurring 0-3m off the bottom, and with a slight temperature gradient of 1-5 °C developing between the epilimnion and hypolimnion. Stratification can occur between mid-May and early September, but is usually observed in June and August. It is ephemeral and can last 1-10 days in various areas of the basin (Bartish 1984).

Stratification occurs in one of two ways. The first is during a period of calm, warm weather (Britt 1955a, Carr *et al.* 1965, Bartish 1984). The second is caused by hypolimnetic or mesolimnetic water spilling over from the central basin into Pigeon Bay, west of Point Pelee, under the influence of strong westerly winds (Bartish 1984, 1987). Both of these phenomena have resulted in anoxic conditions in the hypolimnion in the western basin (Britt 1955a, Carr *et al.* 1965, Britt *et al.* 1968, Hartman 1972, Bartish 1987).

Anoxia was not recorded until the early 1950s. Little or no oxygen depletion was recorded in the hypolimnion between 1929 and 1942 (Wright 1955, Chandler 1940, 1944, Chandler and Weeks 1945). Eutrophication of the western basin increased the sediment oxygen demand that reduced the hypolimnetic oxygen and allowed anoxia to occur (Carr *et al.* 1955). Rathke (1984) estimated that anoxia, based on the biological oxygen demand of the sediment at that time, would result in 3-4 days during a stratification event in the western basin.

Hexagenia and anoxia

Anoxia from stratification, as mentioned above, is detrimental to *Hexagenia* nymphs. This is due to their low tolerance for oxygen concentrations below 1.0 mg O_2 /L water, where death will occur within 30-48 h (Hunt 1953). Growth is also reduced at low oxygen

concentrations (Winter 1994).

There is little knowledge of the effects of low oxygen levels on *Hexagenia* embryos (Fremling 1967). The embryos rest on the benthic sediments until they hatch, and can, therefore, be exposed to short term anoxic events (1-4 days) from stratification. If the embryos survive periods of anoxia, they could allow for the nymph populations to recover. This was proposed by Britt (1955b) to explain the reappearance of the nymphs in 1954, after the 1953 crash.

The embryos can also be exposed to anoxia by becoming buried in the sediment. Oxygen levels fall to 0 mg O_2 /L 2-2.5 mm below the surface of the sediment (Jørgensen and Revsbech 1985, Gundersen and Jørgensen 1990, Ventling-Schwank and Livingstone 1994). Burial would lead to the embryos being in anoxic conditions for different lengths of time. Survival of embryos during burial could lead to an egg bank such as those observed in species of copepods (De Stasio 1989, Hairston *et al.* 1995). Embryos that resurface to the sediment-water interface would subsequently hatch. Embryos emerging at different times, and hatching, could lead to the multiple cohorts often found in nymph populations (Heise *et al.* 1987). It is possible that the different cohorts found could be related to storm events that would be responsible for mixing sediments and re-exposing the embryos to the sediment-water interface.

In this thesis, I demonstrate, by exposing *Hexagenia* embryos to anoxic conditions, that *Hexagenia* embryos will tolerate anoxic events. Because of the absence of oxygen, I anticipated that development would probably be delayed and survivorship would decrease the longer the exposure to anoxic conditions. The ability of *Hexagenia* to survive anoxia over the

temperature range in the western Lake Erie and at different embryological developmental stages will allow the embryos to hatch when they return to normoxic condition. Short term (days) survival of the embryos will allow the *Hexagenia* population to recover if anoxic conditions from a stratification event occur. Longer term survival (weeks to months) will allow the embryos to survive periods of burial. Embryos would re-emerge to the sediment surface at different times, e.g. after a storm event, hatch, and this could create the different cohorts that can be found in a population.

The main objective of my study was to examine the effects of anoxia on the hatching of *Hexagenia* embryos. I studied the effects of anoxia on the survival and rate of development of the embryos at different temperatures (Chapter One) and at different development stages (Chapter Two). Implications of the results were then related to the ecology *Hexagenia* nymphs populations, in terms of distribution in the western basin of Lake Erie, and dynamics of the multiple cohorts found. The purpose of Chapter Three was to determine if anoxic effects, or sediment contaminant effects on the embryos were inhibiting the establishment of nymphs in some areas of the western basin.

CHAPTER ONE: The effects of anoxia at different temperatures on *Hexagenia* mayfly eggs

Introduction

Hexagenia mayfly eggs are oviposited on the water surface by female imagoes after mating. The eggs settle to the lake bottom, where embryonic development begins. Here, the embryos can experience changes in the epibenthic temperature and oxygen levels.

The effects of temperature on development and survival of the embryos have been well studied (Hunt 1953, Fremling 1967, Friesen *et al.* 1979, Wright *et al.* 1982, Heise *et al.* 1987) but there is only incidental knowledge on the effects of anoxia on the embryos (Fremling 1967).

The rate of embryonic development is temperature dependent (Hunt 1953, Fremling 1967, Friesen *et al.* 1979, Wright *et al.* 1982, Heise *et al.* 1987). In laboratory studies, Friesen *et al.* (1979) determined *H. rigida* eggs do not hatch at 8 or 36 °C and that there is a hyperbolic relationship between temperature and time to hatching. Hatching viability was reduced below 16 °C and above 32°C (Friesen *et al.* 1979). Even though *Hexagenia* embryos do not hatch at temperatures below 8 °C, they remain viable and will hatch when the temperature is increased (Friesen *et al.* 1979, Giberson and Rosenberg 1992b). This implies that eggs could overwinter (Heise *et al.* 1987, Giberson and Rosenberg 1992b).

As indicated in the general introduction, developing *Hexagenia* embryos in the western basin of Lake Erie can become exposed to anoxic events by one of two ways:

hypolimnetic stratification, and burial beneath the sediment. Anoxia due to intermittent stratification is a short term event, typically lasting 1-4 days in western Lake Erie (Bartish 1984). An anoxic event from stratification eradicated the nymph population in western Lake Erie in 1953 (Britt 1955a). Britt (1955b) hypothesized that the reappearance of *Hexagenia* in 1954 was in part due to eggs surviving and hatching once conditions returned to a normoxic state.

Burial of the eggs beneath the sediment would lead to longer duration anoxia. Anoxia occurs 2-2.5 mm below the surface of the sediment as a consequence of bacterial respiration or sediment oxygen demand (SOD) (Jørgensen and Revsbech 1985, Gundersen and Jørgensen 1990, Ventling-Schwank and Livingstone 1994). Eggs buried by sedimentation can subsequently be re-exposed by storm turbulence. Egg burial could lead to an egg bank, as in the case of copepods (De Stasio 1989, and Hairston *et al.* 1995). Other species, such as *Artemia* (Clegg 1997), are also capable of long term survival of anoxic events at the egg stage. Groups of eggs resurfacing and hatching at different times could lead to the multiple cohorts found in *Hexagenia* populations (Heise *et al.* 1987).

The above examples indicate that the survival of the embryos over anoxic periods may be important to the long term survival and size structure of nymph populations. The purpose of this study is to examine the effects of temperature and oxygen on *Hexagenia* embryos. Although studies have been done on temperature and one study on anoxia, no studies have been done on the combined effects of both. The effects of anoxic and normoxic conditions, over western Lake Erie's temperature range, on the development and viability of *Hexagenia* embryos are examined here.

I expected that the development of the eggs should be temperature dependent in normoxic conditions (Hunt 1953, Fremling 1967, Friesen *et al.* 1979, Wright *et al.* 1982, Heise *et al.* 1987). The relationship between temperature and embryonic development for many insects has been explained with a power function (*Argia vivida* (Odonata): Leggott and Pritchard 1985; *Ecdyonurus spp.* (Ephemeroptera): Humpesch 1960), exponentionally (*Dinocras spp.* (Plecoptera): Zwick 1996), and by a hyperbolic function (*Ephemerella ignita* (Ephemeroptera): Elliott 1978). Previously, the relationship between temperature and time to first hatching in *Hexagenia* has been reported to be hyperbolic (Friesen *et al.* 1979). Also, the relationship is linear between the developmental rate and temperature for *Hexagenia* eggs (Hunt 1953, Flattum 1963, Freisen *et al.* 1979, Wright *et al.* 1982). These trends should be maintained in my study. Anoxia should delay the development of the eggs at all temperatures, as it does in other organisms (e.g. copepods: Marcus and Lutz 1992, *Artemia*: Clegg 1997).

Viability, in normoxic conditions, should decrease at the lower temperatures, whereas under anoxic conditions I expect that higher temperatures would have a greater effect on viability. The effects of temperature and anoxia should become more pronounced the longer the eggs are exposed to the different conditions.

Methods

This experiment was initially done during the summer of 1997 and was repeated in 1998.

Egg collection

Hexagenia eggs, in both years, were collected from female imagoes at Colchester Harbour, Ontario (41°59' N; 82°56' W), Lake Erie. The adults are easily collected at dusk, when they gather around the lights of the harbour. Resting female imagoes were grasped by the wings and placed in a 2-L polyethylene soil bag containing aerated, dechlorinated water. When placed on the water's surface, the females oviposited their eggs into the water. Eggs from 25-27 females were collected in each bag. On average, a female releases 4000 eggs (Hunt 1953). Female imagoes were removed from the bags the following morning, and placed into 70% ethanol. The eggs were collected from a mixture of *Hexagenia limbata* and *Hexagenia rigida* female imagoes.

In 1997, due to poor emergence of female imagoes, 2 sampling dates were needed to collect 5 replicate bags of eggs. On 27 July 1997, two bags were collected and designated as replicates A and B. The second set of bags, replicates C, D and E, were collected the following day on 28 July.

In 1998, all five replicate bags of eggs were collected on 21 June. This was during a period of mass emergence of *Hexagenia* subimagoes and imagoes (personal observation, Schmidt 1998, L.D. Corkum, University of Windsor, Windsor, ON, pers. comm.).

Experimental design

To examine the effects of oxygen and temperature on *Hexagenia* mayfly embryos, a 2 (oxygen) x 4 (temperature) x 6 (time in treatment) factorial design was used (n =5 replicates/treatment) (Figure 1.1). For a replicate, approximately a 100 eggs were initially placed in 20 mL scintillation vials containing either dechlorinated anoxic water (< 1.0 mg O_2/L) or dechlorinated normoxic water (6-8 mg/L). Vials for both the anoxic and normoxic conditions were placed randomly in incubators set at one of four temperatures: 5, 8, 16 and 20 °C. This encompasses the range of values that the eggs would be exposed to at the bottom of the western basin of Lake Erie (Schertzer *et al.* 1987). On days 0, 1, 4, 7, 15, and 28 five replicate vials for each oxygen and temperature treatment were removed. The eggs were then transferred to petri dishes containing aerated dechlorinated water at 20 °C to allow for hatching. Day 0 removals served as treatment controls. Day 1 and 4 removals were used to examine effects of short term anoxia due to stratification (Bartish 1984). For longer term anoxia day 7 - 28 removals were used.

Long term viability in anoxic conditions was further examined by placing one set of eggs from each replicate in anoxic conditions at 20 °C for 245 d (8 mo.) in 1997.

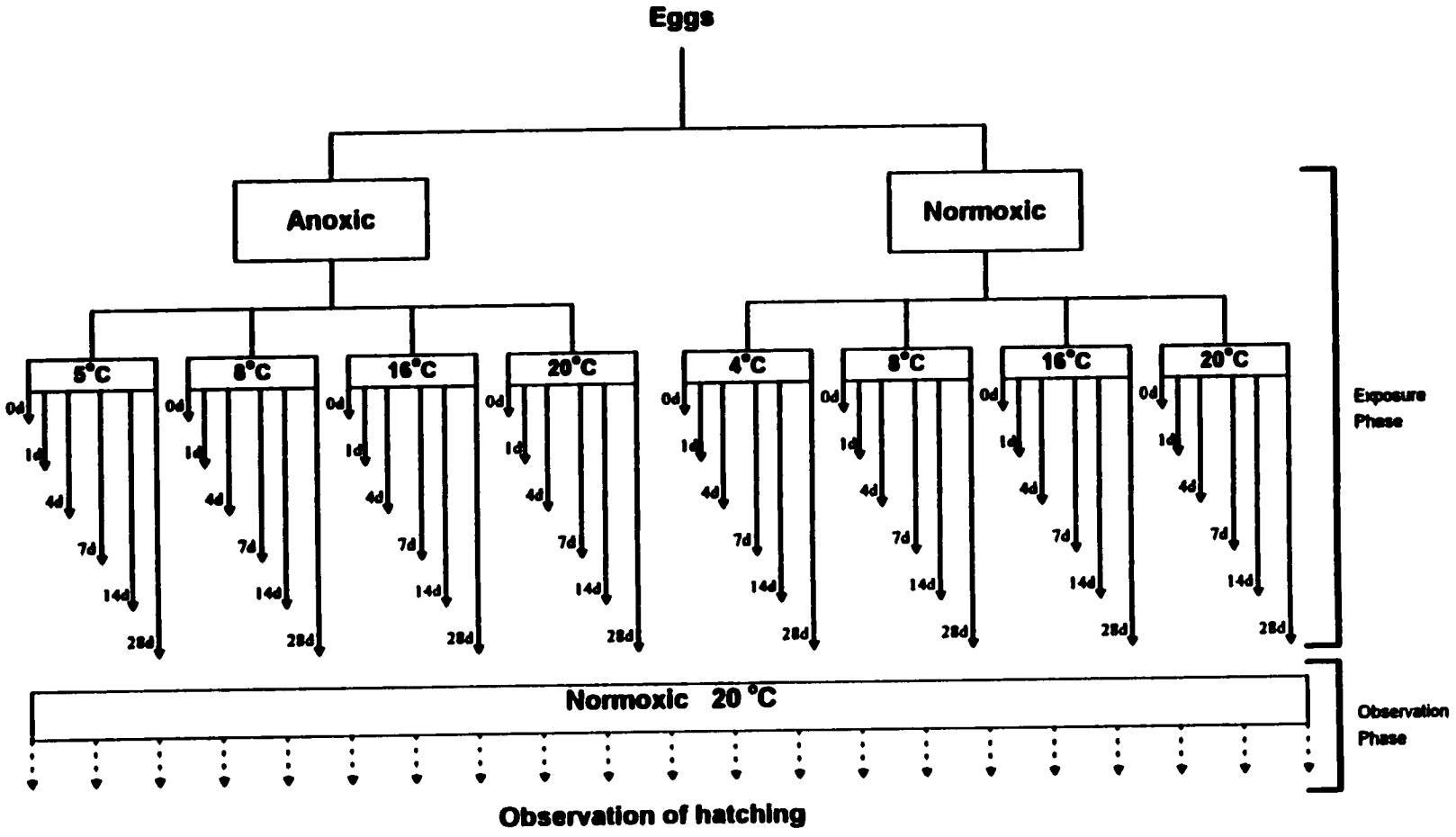


Figure 1.1: Experimental design for *Hexagenia* embryological development

Experimental procedure

The eggs, in 1997, were placed into treatment two days after collection. In 1998, the eggs were placed into treatment the day after collection.

Anoxic treatments

Water for the anoxic treatments was produced by bubbling a 1000 mL aliquot of 20 °C carbon filtered dechlorinated tap water with 99.95% nitrogen gas, for at least 1/2 h water before use.

The eggs had the aerated water, in which they were collected, filtered off. They were then rinsed with and placed into anoxic water, before being placed into the treatment vials (A.H. Warner, University of Windsor, Windsor, ON, pers comm.). This minimized the introduction of oxygen into the vials.

To place the eggs in anoxic conditions, 20 mL scintillation vials were filled over the top, to prevent air bubbles when capped, with the anoxic water. Immediately after a vial was filled with water, a drop of the eggs from a Pasteur pipette was added. The vial was then capped and sealed with Parafilm.

Normoxic treatments

For the normoxic treatments, the vials were filled following the same procedure as above using 20 °C aerated, carbon filtered dechlorinated, tap water. The eggs were washed and rinsed with aerated, dechlorinated water before they were added to the vials.

Temperature treatments

Once the anoxic and normoxic vials for one replication had been filled, they were placed in incubators set at nominal temperatures of 5, 8, 16 and 20 °C. To dampen slight temperature changes in the incubators caused by heating/cooling cycles, the vials were immersed in pans of water.

Maintenance of normoxia

The *Hexagenia* embryos consume oxygen as they develop. Therefore, the water in the vials was replaced on a regular basis to ensure that the normoxic treatments did not become hypoxic (<6.5 mg/L). In 1997, the water was changed every 4 d. Based on the results of an oxygen consumption study of the embryos (Appendix II), I decided, in 1998, to replace the water every 2 d.

The water in each vial was partially replaced with dechlorinated normoxic, or dechlorinated anoxic water that had been cooled in the incubators to the appropriate temperature. Half the water in the vial was pipetted off, carefully, to prevent the loss of any eggs. The vial was then filled with the fresh water. While the water was being changed, the vials were kept in water baths at the appropriate temperature and monitored to ensure their temperature did not vary more than ±1 °C . The vials were resealed and placed back into their respective treatment incubators.

For the anoxic vials, the water exchange was conducted in an anaerobic chamber to ensure that no oxygen was introduced.

Removal from treatment conditions for hatching

Vials were to remain in treatment for 0, 1, 4, 7, 15, and 28 d. One replicate vial was removed on each of the designated days. Day 0 treatment vials were removed from the treatment 1 h after being placed in the incubators.

Upon removal from the incubators, the vials were placed at 20 °C and remained there for 1-2 h until their temperature was 20 °C. This allowed for O_2 measurements to be taken. The vials were opened, one at a time in random order. The oxygen was immediately measured using a Clark Style O_2 microelectrode (Diamond General Inc., Ann Arbor, MI). The O_2 meter was calibrated 0-100 % saturation, using aerated and anoxic dechlorinated water. After the oxygen had been measured, most of the water in a vial was carefully removed using a pipette. Approximately the last 2 mL, containing the eggs, was left in the vial. The eggs were then rinsed into a 60 mm diameter x 15 mm deep plastic petri dish with 20 °C aerated dechlorinated water. Using the petri dishes allowed me to monitor the embryos under the dissecting microscope. The petri plates were covered and remained at 20 °C.

Petri plates containing embryos were examined daily for hatching. When nymphs were found they were counted and removed using a Pasteur pipette. Monitoring of the eggs continue until no further hatching was likely to occur, as indicated by the eggs browning or internal deterioration (Friesen *et al.* 1979). The plates were topped up with aerated dechlorinated water at 20 °C as needed.

Data analysis

Daily counts of nymphs for each replicate at each treatment were used to determine the time to first, and time to midpoint hatching. Time to first hatching was determined by the initial 7 % of hatching in each treatment. Seven percent of hatching was based on percentage hatching that one egg was equivalent to in the vial with the least number of eggs (14 eggs). Midpoint hatching is the time by which 50% of the total number of eggs had hatched. These measures were used to determine the effects of the treatments on the development of the eggs. Total counts of hatched nymphs were used to determine the viability of the embryos (no. hatched/ no. in a vial).

The effects of the oxygen (normoxia and anoxia), temperature, days in treatment, their interactions, replication and number of eggs per vial on both development and viability were analyzed by linear regression (SAS Institute Inc. 1985).

Separate analyses for each year's data were performed to estimate experimental effects of the 3 dependent variables: time to 7 % hatching, to 50 % hatching of total and viability. Independent variables were days in treatment, treatment temperature, treatment oxygen (normoxic/anoxic), and their interactions. The number of eggs per vial and the replicates were included as covariates. Linear, log-linear, and log-log combinations of transformations were examined. The regression model that gave the most consistently high coefficients of determination were ultimately used.

Results

In 1997, there were problems with two of the incubators keeping the temperatures to which they were set as was later ascertained from continuous temperature logger recordings. Therefore, the four treatment temperatures were 4.5, 11 ,11, and 20 °C instead of 5, 8, 16, and 20 °C, respectively. In 1998, the actual treatment temperatures were 5, 8.8, 15, and 20 °C.

The number of eggs per vial ranged from 14 - 319, with a mean ± SD of 69.6 ± 56.4, in 1997. In 1998, the number of eggs per vial ranged from 39-553, with a mean ± SD of 131.6 ± 61.9. The frequency distributions of the number of eggs per vial for both years are in Figure 1.2.

In 1997, the oxygen level in the vials, at the end of treatment (final oxygen), was variable for the normoxic treatments, ranging from 0-9.1 mg/L. In 1998, the final oxygen concentrations in the vials ranged from 6.9-9.7 mg/L. Even though some of the Day 0 anoxic treatments had a residual oxygen level as high as 0.8 mg/L, by Day 1 the oxygen level was 0 mg/L, except in the 4 °C treatments. Many of the 4 °C anoxic treatment vials did have a drop in the oxygen level from approximately 0.5 mg/L to 0 - 0.1 mg/L after the first water change in the anaerobic chamber.

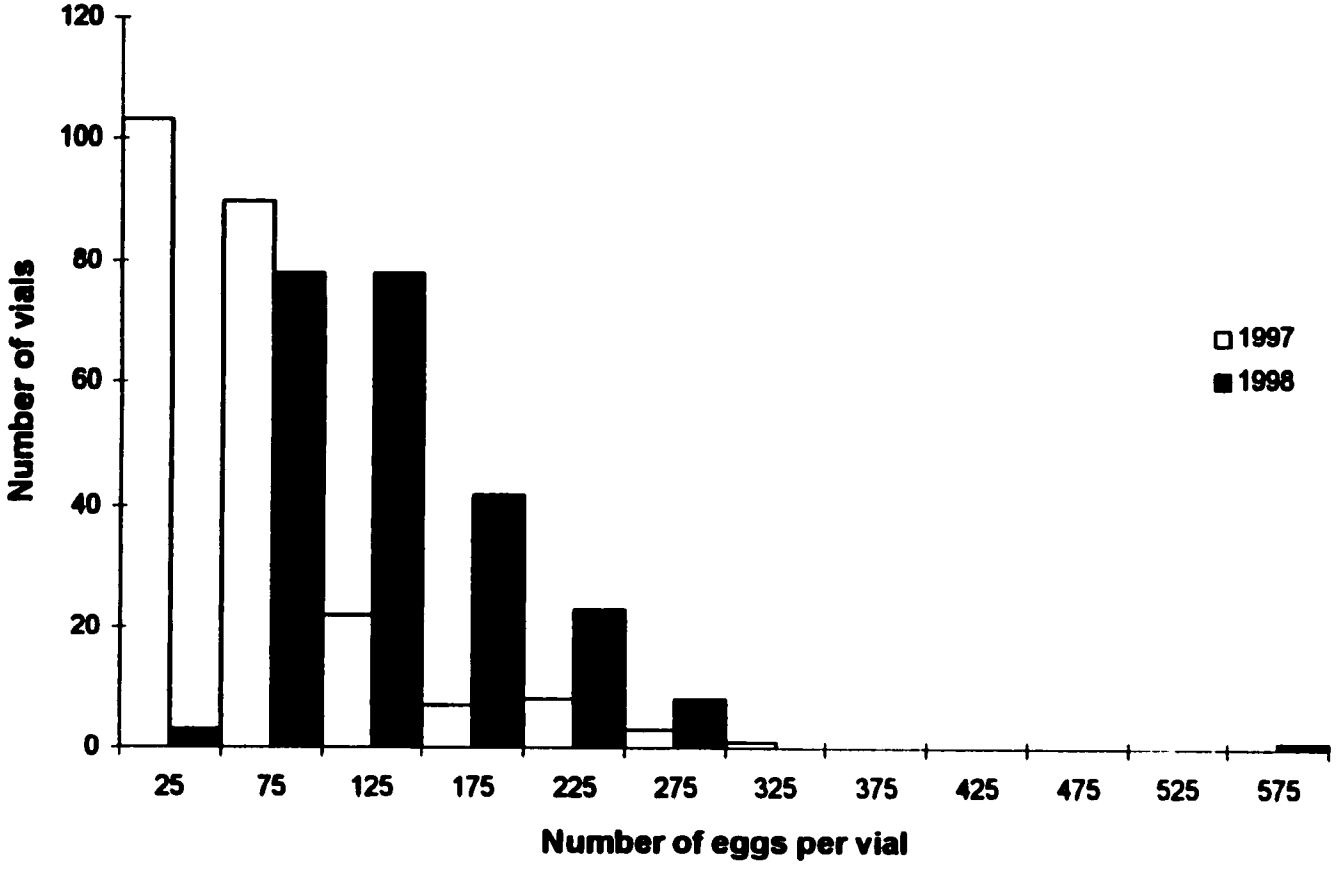


Figure 1.2: Frequency distribution of the number of *Hexagenia* eggs per vial in 1997 and 1998.

Viability

Viability, measured as the percentage of the *Hexagenia* embryos hatching, decreased with increasing time in both normoxic and anoxic treatments (Table 1.1, Figure 1.3 and 1.4). In 1997, the model that best described the effects of oxygen, treatment temperature, days in treatment, their interaction, number of eggs and replicate on the viability was to regress the independent variables against log viability ($R^2 = 0.62$; Table 1.2). In 1998, the best model was described by log transforming temperature and time in treatment ($R^2 = 0.70$; Table 1.3).

Time in treatment had a significantly negative effect on the viability ($p < 0.05$, in 1997; Table 1.2 and $p < 0.0001$, in 1998; Table 1.3). As main effects, neither temperature nor anoxia/normoxia influenced viability (Table 1.2). In 1998, the embryos that were in treatment for longer than 7 d had a lower viability after being in the normoxic treatments than in the anoxic treatments at all temperature treatments except 4 °C (Figure 1.4b-d). In 1997, this was only observed for the 11 °C treatment embryos (Figure 1.3b). This was reflected in a significant ($p < 0.05$) oxygen x temperature x time in treatment interaction (Table 1.2 and 1.3). There was a positive relationship between viability and the number of eggs per vial in 1998 (Table 1.3), but not in 1997 (Table 1.2)

The overall viability in 1997 was low, with an initial percent hatching (mean ± SD %), in the control embryos (embryos in treatment for 0 d), of 45.6 ± 9.2 %. In 1998, the viability (mean ± SD %) was 80.8 ± 7.7 %, in the control treatments.

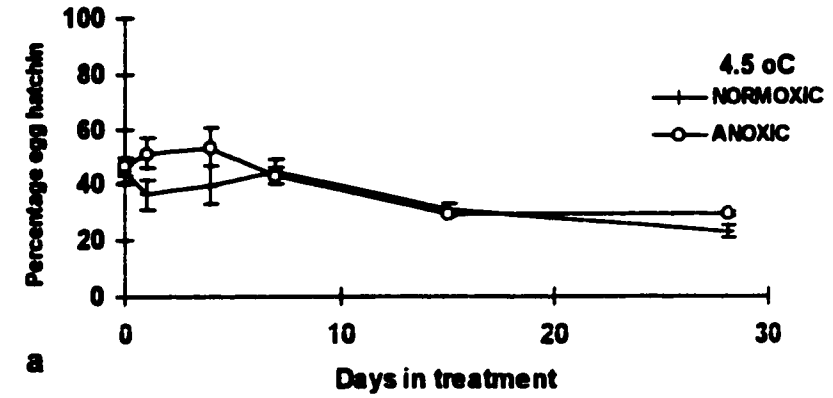
Long term effects of anoxia

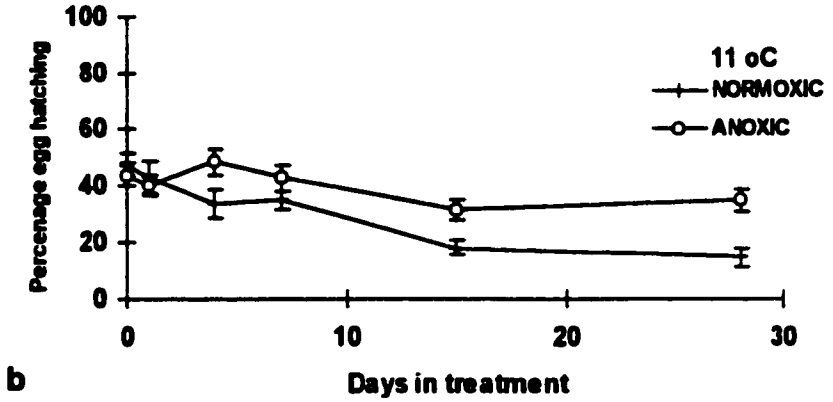
In 1997, the long term effects of anoxia were examined by keeping one set of embryos in anoxia at 20 °C for 245 d (8 mo). The mean viability (± SD), after the 8 mo. in anoxia, was 16.4 ± 7.10 %. Viability decreased as a log function of time in anoxia according to equation (1.1) (Figure 1.5).

$$\log(\text{viability}) = 1.67 - 0.0896\,[\log(\text{time in anoxia})]^2 \quad (R^2 = 0.73) \qquad (1.1)$$

Table 1.1: Viability (Mean ± SD, n= 5) of *Hexagenia* embryos in anoxic and normoxic treatments at different temperature treatments (°C), for different time periods in treatment (d).

			Days in Treatment					
		Treatment	0	1	4	7	14	28
Year	Oxygen treatment	Temperature (°C)	% hatch ± SD	% hatch ± SD	% hatch ± SD	% hatch ± SD	% hatch ± SD	% hatch ± SD
1997	normoxic	4.5	44.4 ± 13.9	36.4 ± 16.7	39.9 ± 3.55	44.4 ± 13.9	30.7 ± 9.64	23.1 ± 4.86
		11	47.4 ± 8.42	43.0 ± 19.5	33.7 ± 13.6	34.9 ± 6.89	18.1 ± 5.93	14.8 ± 4.50
		11	43.1 ± 11.3	47.2 ± 8.71	48.5 ± 8.95	41.0 ± 8.28	23.6 ± 6.08	16.2 ± 8.81
		20	41.8 ± 9.64	48.0 ± 12.2	40.0 ± 6.45	43.8 ± 10.9	21.7 ± 5.02	NA
	anoxic	4.5	46.7 ± 7.21	51.3 ± 11.8	53.4 ± 15.4	43.3 ± 9.57	29.5 ± 4.81	29.6 ± 4.86
		11	43.9 ± 9.39	40.0 ± 10.6	48.3 ± 13.3	42.5 ± 7.02	31.6 ± 4.40	34.7 ± 11.3
		11	46.4 ± 9.00	38.9 ± 7.49	43.8 ± 7.50	44.4 ± 13.3	35.6 ± 12.7	29.9 ± 5.85
		20	51.5 ± 5.83	44.8 ± 3.7	44.7 ± 5.91	42.5 ± 9.14	27.7 ± 2.63	30.9 ± 2.08
1998	normoxic	5	80.4 ± 7.64	81.7 ± 3.65	69.5 ±13.24	57.6 ± 9.59	58.6 ± 12.25	32.1 ±14.72
		8.8	82.9 ± 8.20	80.6 ± 3.36	80.3 ± 3.52	70.5 ± 5.96	53.7 ± 9.92	37.0 ± 13.26
		15	82.4 ± 4.52	81.3 ± 3.99	67.4 ±9.07	53.8 ± 4.14	45.4 ± 9.83	42.6 ± 11.69
		20	82.7 ± 9.30	79.5 ± 6.03	73.4 ± 6.90	59.3 ± 14.24	48.7 ± 10.06	NA
	anoxic	5	83.8 ± 5.89	80.3 ± 9.02	68.9 ± 11.92	67.0 ± 8.27	62.6 ± 13.26	47.8 ± 19.08
		8.8	76.8 ± 10.10	83.7 ± 6.56	70.9 ± 5.56	65.7 ± 10.48	64.3 ± 10.41	66.7 ± 8.04
		15	81.7 ± 6.14	80.0 ± 5.40	67.1 ± 11.42	60.3 ± 11.04	61.3 ± 15.08	56.6 ± 6.36
		20	75.5 ± 9.16	85.3 ± 4.66	65.3 ± 10.08	72.4 ± 2.28	66.0 ± 8.89	63.8 ± 7.06





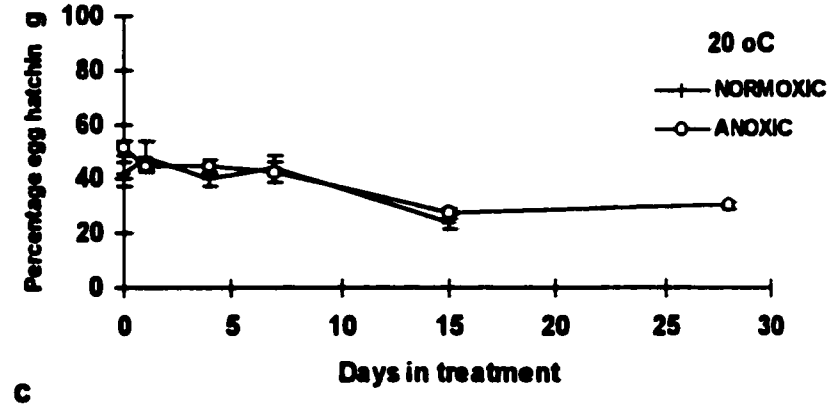


Figure 1.3: Viability of *Hexagenia* embryos (% ± SE, n=5) in normoxic water, after eggs were in anoxic and normoxic conditions at a) 4.5 °C , b) 11 °C , and c) 20 °C for 0, 1, 4, 15, and 28 d. 1997 data.

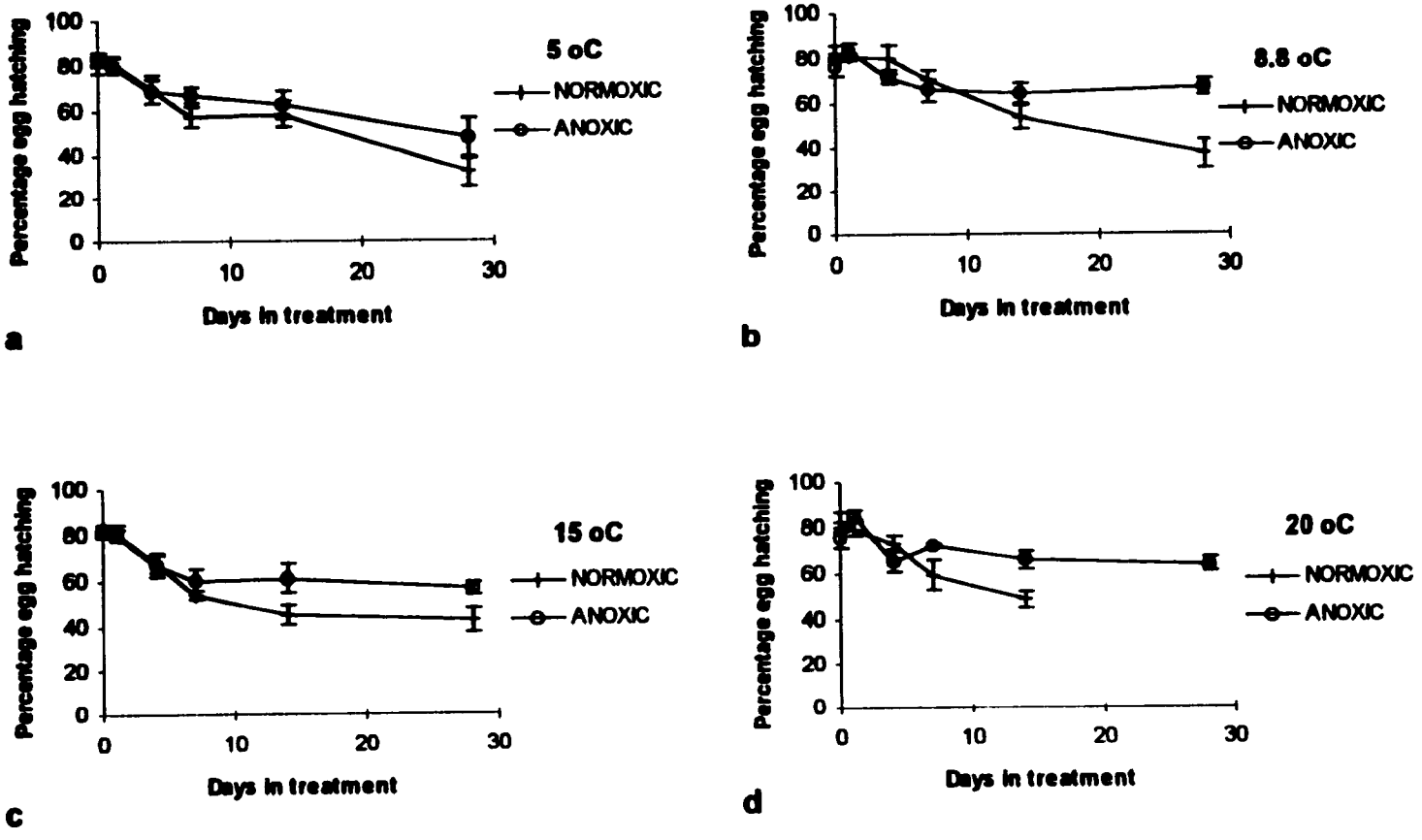


Figure 1.4: Viability of *Hexagenia* embryos (% ± SE, n=5) in normoxic water, after eggs were in anoxic and normoxic conditions at a) 5 °C , b) 8.8 °C , c) 15 °C and d) 20 °C for 0, 1, 4, 15, and 28 d. 1998 data.

Table 1.2: Summary of the linear regression for the effects of oxygen treatment, temperature treatment, time in treatment, their interactions, number of eggs per vial, and replicate (r1-r4), and on the log viability of *Hexagenia* embryos for 1997.

Regression

Variable	DF	Coeff.	SE	R^2
intercept	1	1.6692	0.0353	
oxygen (oxy)	1	-0.0624	0.0455	0.05
time in treatment (time)	1	-0.0060 *	0.0023	0.37
treatment temperature (temp)	1	-0.0005	0.0024	0.00
oxy x time	1	-0.0009	0.0037	0.06
oxy x temp	1	0.0052	0.0036	0.00
temp x time	1	-0.0001	0.0002	0.01
oxy x time x temp	1	-0.0008 *	0.0003	0.02
number eggs per vial	1	0.0003	0.0001	0.00
r1	1	-0.0692	0.0235	
r2	1	-0.1118	0.0232	0.11
r3	1	0.0413	0.0232	
r4	1	0.0251	0.0232	

Analysis of Variance

	DF	SS	MS	F
model	12	4.533	0.378	30.007***
error	222	2.794	0.013	
total	234	7.327		

$R^2 = 0.6186$

* : $p < 0.05$
** : $p < 0.001$
*** : $p < 0.0001$

Table 1.3: Summary of the linear regression for the effects of oxygen treatment, temperature treatment, time in treatment, their interactions, number of eggs per vial, and replicate, and on the viability of *Hexagenia* embryos for 1998.

Regression

Variable	DF	Coeff.	SE	R^2
intercept	1	85.490	7.255	
oxygen (oxy)	1	5.781	9.623	0.02
time in treatment (time)	1	-14.103***	3.221	0.52
treatment temperature (temp)	1	-2.948	2.725	0.0
oxy x time	1	-2.767	4.690	0.05
oxy x temp	1	0.227	3.909	0.0
temp x time	1	2.854	1.305	0.0
oxy x time x temp	1	-1.630*	1.934	0.0
number eggs per vial	1	0.047***	0.011	0.06
r1	1	-6.0577*	1.948	
r2	1	-4.482	2.005	0.03
r3	1	-4.051	2.049	
r4	1	2.018	1.889	

Analysis of Variance

	DF	SS	MS	F
model	12	42308.467	3525.706	42.184***
error	222	18554.400	83.578	
total	234	60862.867		

$R^2 = 0.6951$

* : $p < 0.01$
** : $p < 0.05$
*** : $p < 0.001$

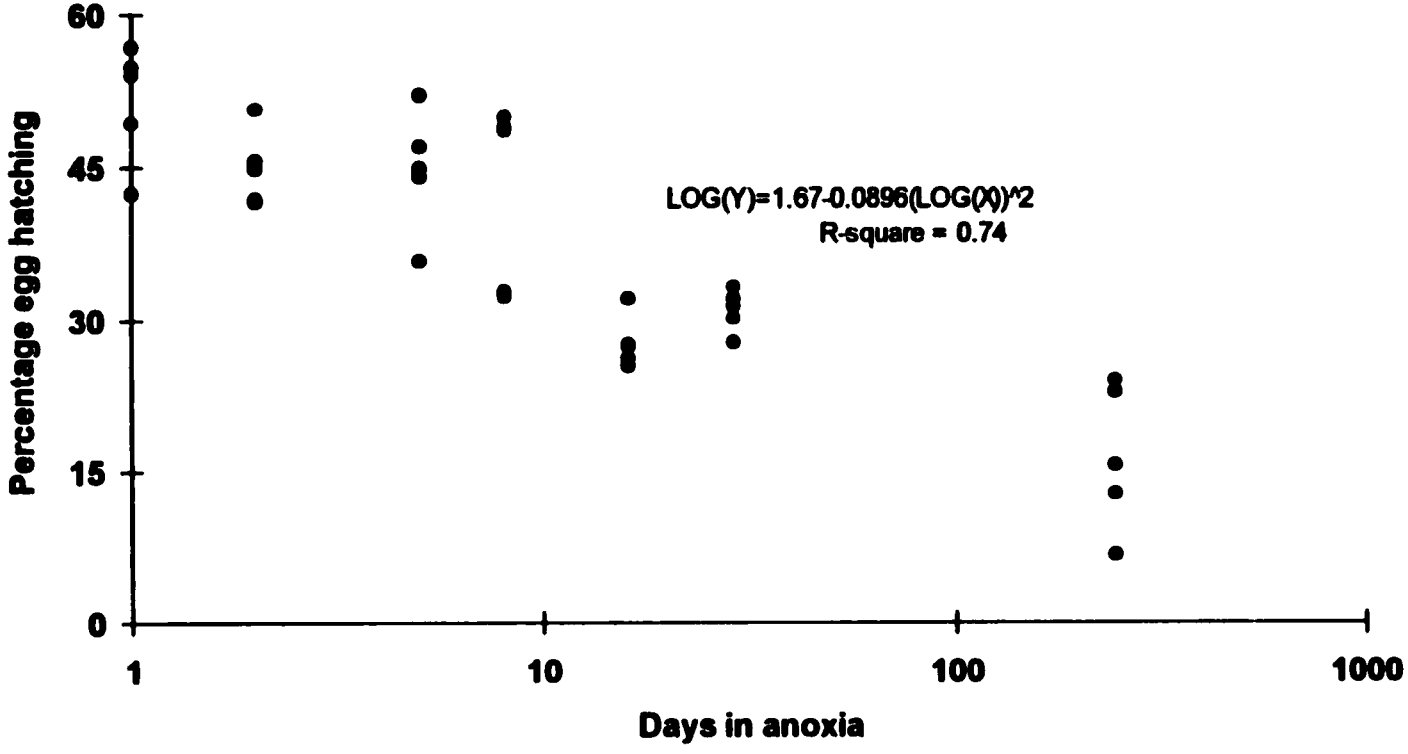


Figure 1.5: Long term viability of *Hexagenia* embryos (percentage hatching) at 20 °C in normoxic conditions after eggs were stored in anoxic water at 20 °C

Development

Preliminary analysis of variance (not shown) indicated that there were complex interactions between oxygen and other main effects on the development. Consequently, separate regressions were performed to predict hatching times for normoxic and for anoxic treatments.

Time to first hatching (mean ± SD), for embryos in the controls (Day 0) under normoxic conditions at 20 °C, was 13.7 ± 0.6 d in 1997, and 14.1 ± 0.9 d in 1998.

Normoxic

In the normoxic treatments, the time to hatching increased the longer the eggs were held in treatment as seen with time to 7% hatching (Table 1.4) and time to 50% (Table 1.5, Figure 1.6a and 1.7a). Time in treatment was a main factor that significantly ($p < 0.0001$) affected the time to hatching and explained most of the variability in the time to hatching among the treatments (partial $R^2 = 0.93$ for 1997 and partial $R^2 = 0.88$ for 1998) both years (Table 1.6 & 1.8 for 1997; Table 1.7 & 1.9 for 1998).

Log treatment temperature, both alone and in the log treatment x time in treatment were significant in predicting the time to hatching. Log temperature had a lesser effect on the time to hatching than its interaction with time in treatment. The interaction was very significant ($p < 0.0001$) in both years for both time to 7% hatching (Table 1.6 and 1.7) and 50% of total hatching (Table 1.8 and 1.9). The longer the eggs were held at the lower temperatures the longer the embryos took to hatch (Table 1.4 and 1.5, Figures 1.6a and 1.7a).

The 4 °C, 11 °C (1997) and 9 °C (1998) treatments showed a delay in hatching equal

Table 1.4: Mean ± SD for days to 7% hatching for *Hexagenia* embryos in anoxic and normoxic treatments at different temperature treatments (°C), for different time periods in treatment (d).

Year	Oxygen treatment	Treatment temperature (°C)	Days in Treatment 0	1	4	7	14	28
			7 % hatch ± SD	7 % hatch ± SD	7 % hatch ± SD	7 % hatch ± SD	7 % hatch ± SD	7 % hatch ± SD
1997	normoxic	4.5	14.0 ± 0.00	15.4 ± 0.55	18.2 ± 0.45	20.8 ± 0.45	29.4 ± 0.55	43.2 ± 0.45
		11	14.3 ± 0.50	14.6 ± 0.55	17.4 ± 0.55	20.2 ± 0.45	27.0 ± 0.71	40.2 ± 0.45
		11	14.2 ± 0.45	14.0 ± 0.00	16.6 ± 0.55	19.4 ± 0.55	27.0 ± 0.71	40.6 ± 0.55
		20	13.6 ± 0.55	13.6 ± 0.55	15.2 ± 0.45	15.8 ± 0.84	18.8 ± 0.96	NA
	anoxic	4.5	14.0 ± 0.00	14.4 ± 0.55	18.2 ± 0.84	21.0 ± 0.00	29.6 ± 0.55	43.0 ± 0.71
		11	14.0 ± 0.00	14.6 ± 0.55	17.8 ± 0.45	20.0 ± 0.71	28.8 ± 0.84	41.5 ± 0.58
		11	14.4 ± 0.55	14.8 ± 0.45	17.8 ± 0.45	20.4 ± 0.55	29.0 ± 1.00	42.0 ± 0.00
		20	14.0 ± 0.00	14.2 ± 0.45	17.2 ± 0.45	20.2 ± 0.45	28.8 ± 0.84	41.6 ± 0.55
1998	normoxic	5	15.0 ± 0.00	15.4 ± 0.55	19.2 ± 0.84	22.4 ± 0.89	31.2 ± 1.64	46.2 ± 1.30
		8.8	15.0 ± 0.00	15.2 ± 0.45	18.6 ± 0.89	21.6 ± 0.55	28.8 ± 0.45	41.8 ± 0.84
		15	15.0 ± 0.00	15.4 ± 0.55	17.8 ± 0.45	19.6 ± 0.55	25.0 ± 0.71	36.4 ± 0.55
		20	14.8 ± 0.45	14.6 ± 0.55	15.6 ± 0.55	16.0 ± 0.71	18.0 ± 0.00	NA
	anoxic	5	15.0 ± 0.71	15.6 ± 0.55	18.8 ± 0.84	22.4 ± 0.55	31.4 ± 1.34	46.2 ± 2.39
		8.8	15.2 ± 0.84	15.6 ± 0.55	18.4 ± 0.55	22.0 ± 0.00	29.4 ± 0.55	42.8 ± 0.45
		15	15.0 ± 0.71	15.4 ± 0.55	18.2 ± 0.45	21.4 ± 0.55	27.6 ± 0.55	40.8 ± 0.45
		20	15.0 ± 0.71	14.8 ± 0.45	18.0 ± 0.00	20.4 ± 0.55	26.8 ± 0.45	40.4 ± 0.55

Table 1.5: Mean ± SD for days to 50% of total hatching for *Hexagenia* embryos in anoxic and normoxic treatments at different temperature treatments (°C), for different time periods in treatment (d).

Year	Oxygen treatment	Treatment temperature (°C)	Days in Treatment 0	1	4	7	14	28
			50% ± SD	50% ± SD	50% ± SD	50% ± SD	50% ± SD	50% ± SD
1997	normoxic	4.5	15.2 ± 0.45	16.0 ± 0.71	19.0 ± 0.00	22.4 ± 0.55	30.6 ± 0.89	44.0 ± 0.71
		11	15.3 ± 0.50	15.8 ± 0.50	18.4 ± 0.55	21.2 ± 0.45	28.0 ± 0.00	41.0 ± 0.82
		11	15.2 ± 0.45	16.0 ± 0.71	18.2 ± 0.45	20.8 ± 0.84	29.0 ± 0.00	41.8 ± 0.96
		20	15.0 ± 0.71	15.0 ± 0.71	16.4 ± 0.55	17.4 ± 0.55	19.7 ± 2.08	NA
	anoxic	4.5	15.6 ± 0.89	15.6 ± 0.89	19.4 ± 0.55	22.3 ± 0.50	30.8 ± 0.45	44.2 ± 0.45
		11	15.2 ± 0.45	15.6 ± 0.55	19.0 ± 0.00	21.4 ± 0.89	30.4 ± 0.55	43.8 ± 0.96
		11	15.2 ± 0.84	16.6 ± 0.89	19.4 ± 0.55	21.8 ± 0.45	30.4 ± 0.55	43.2 ± 0.84
		20	14.8 ± 0.45	16.2 ± 0.84	19.2 ± 0.84	21.8 ± 0.84	30.6 ± 0.89	42.6 ± 1.82
1998	normoxic	5	15.6 ± 0.24	16.6 ± 0.24	20.6 ± 0.24	24.6 ± 0.51	34.4 ± 1.08	48.2 ± 0.97
		8.8	15.6 ± 0.24	16.6 ± 0.24	19.8 ± 0.20	22.8 ± 0.20	30.6 ± 0.24	43.6 ± 0.40
		15	15.8 ± 0.20	16.6 ± 0.24	19.2 ± 0.37	21.2 ± 0.20	26.6 ± 0.24	38.8 ± 0.37
		20	15.8 ± 0.37	15.6 ± 0.24	16.6 ± 0.24	17.2 ± 0.37	19.2 ± 0.20	NA
	anoxic	5	16.0 ± 0.32	17.0 ± 0.45	20.6 ± 0.68	24.4 ± 0.68	33.8 ± 1.28	48.2 ± 0.97
		8.8	16.2 ± 0.20	16.6 ± 0.24	20.2 ± 0.37	23.2 ± 0.20	30.6 ± 0.24	44.6 ± 0.24
		15	16 2 ± 0.20	16.4 ± 0.24	19.8 ± 0.37	22.4 ± 0.24	29.6 ± 0.24	42.6 ± 0.24
		20	16.2 ± 0.37	16.0 ± 0.32	19.2 ± 0.37	21.6 ± 0.24	28.4 ± 0.24	42.0 ± 0.32

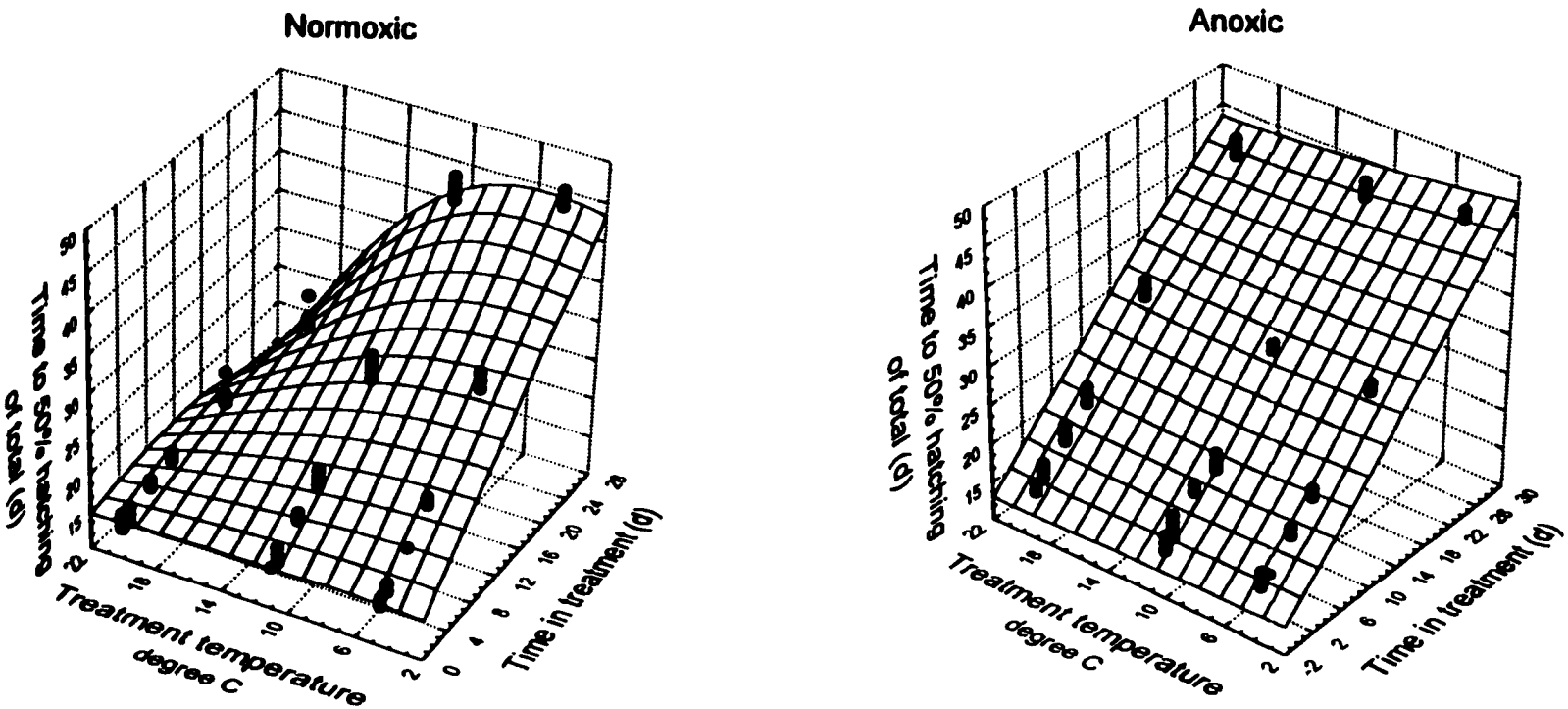


Figure 1.6: Least square surface plot of the effects of days in treatment at different treatment temperatures for a) normoxic and b) anoxic conditions on the time to 50% hatching of the total hatching of *Hexagenia* embryos. 1997 data.

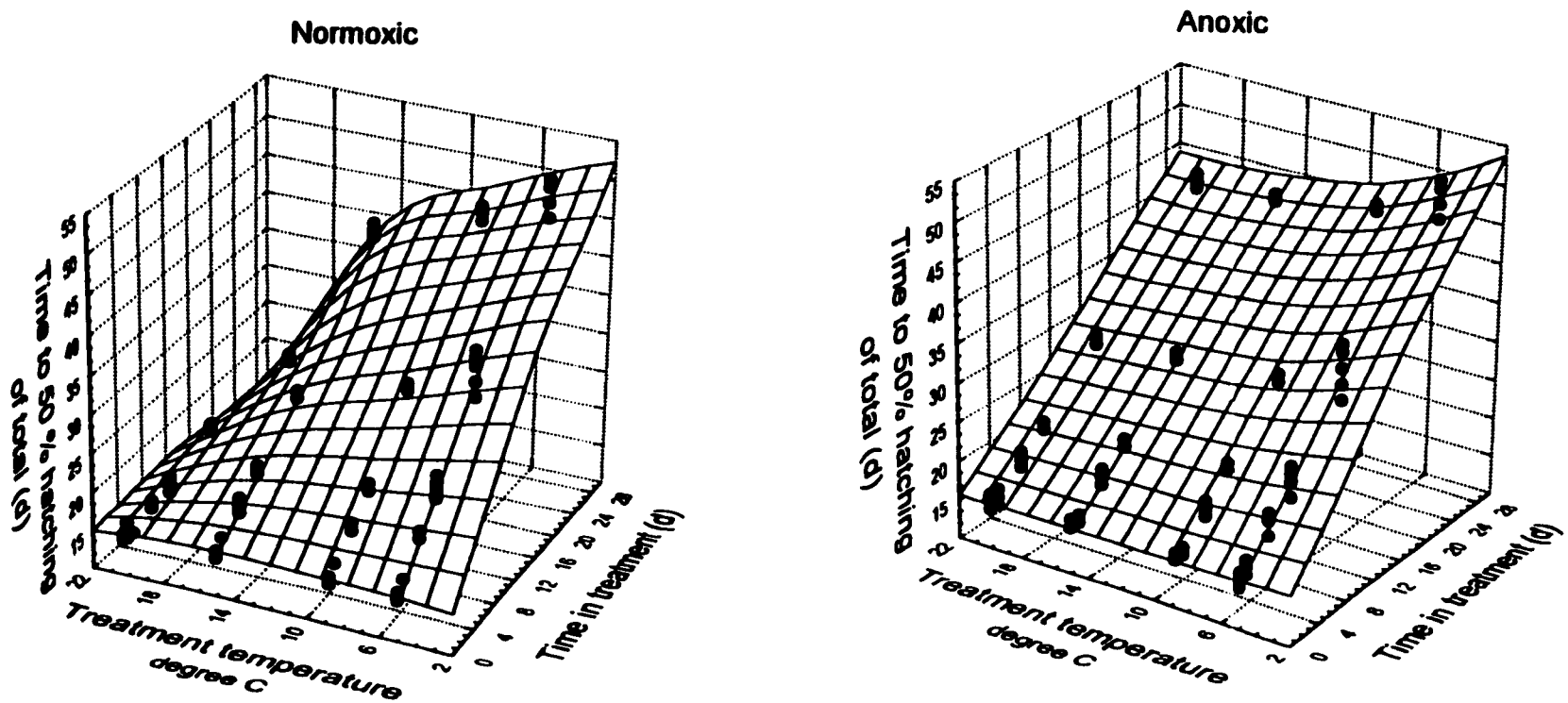


Figure 1.7: Least square surface plot of the effects of days in treatment at different treatment temperatures for a) normoxic and b) anoxic conditions on the time to 50% hatching of the total hatching of *Hexagenia* embryos. 1998 data.

Table 1.6: 1997 normoxic treatments: Summary of the linear regression model of the effects of time in treatment, log treatment temperature, their interaction, number of eggs per vial, and replicates (r1-r4), on the time to 7 % hatching of *Hexagenia* embryos.

Regression

Variable	DF	Coeff.	SE	R^2
intercept	1	15.989	0.968	
time in treatment (time)	1	1.428***	0.082	0.93
log treatment temperature (temp)	1	-1.126**	0.398	0.03
temp x time	1	-0.225***	0.037	0.01
number eggs per vial	1	-0.004	0.004	0.00
r1	1	0.231	0.495	
r2	1	0.280	0.477	0.00
r3	1	-0.044	0.476	
r4	1	0.008	0.477	

Analysis of Variance

	DF	SS	MS	F
model	8	9094.944	1136.868	435.604***
error	106	297.646	2.610	
total	114	9371.590		

$R^2 = 0.9705$

* : $p < 0.01$
** : $p < 0.05$
*** : $p < 0.001$

Table 1.7: 1998 normoxic treatments: Summary of the linear regression model of the effects of time in treatment, log treatment temperature, their interaction, number of eggs per vial, and replicates (r1-r4), on the time to 7 % hatching of *Hexagenia* embryos.

Regression

Variable	DF	Coeff.	SE	R^2
intercept	1	14.414	0.827	
time in treatment (time)	1	1.796***	0.062	0.89
log treatment temperature (temp)	1	-0.819**	0.297	0.05
temp x time	1	-0.393***	0.027	0.04
number eggs per vial	1	0.003	0.002	0.00
r1	1	1.295**	0.403	
r2	1	1.813***	0.423	0.00
r3	1	1.043	0.426	
r4	1	0.838	0.399	

Analysis of Variance

	DF	SS	MS	F
model	8	9139.303	1142.413	6.82.77***
error	106	177.358	1.673	
total	114	9316.661		

$R^2 = 0.9810$

* : $p < 0.01$
** : $p < 0.05$
*** : $p < 0.001$

Table 1.8: 1997 normoxic treatments: Summary of the linear regression model of the effects of time in treatment, log treatment temperature, their interaction, number of eggs per vial, and replicates (r1-r4), on the time to 50 % of total hatching *Hexagenia* embyros.

Regression

Variable	DF	Coeff.	SE	R^2
intercept	1	16.922	1.018	
time in treatment (time)	1	1.433***	0.086	0.93
log treatment temperature (temp)	1	-0.951	0.416	0.02
temp x time	1	-0.232***	0.039	0.01
number eggs per vial	1	-0.002	0.004	0.00
r1	1	0.144	0.521	
r2	1	0.099	0.502	0.00
r3	1	-0.261	0.501	
r4	1	-0.139	0.502	

Analysis of Variance

	DF	SS	MS	F
model	8	8879.707	1109.963	384.246***
error	106	306.200	2.889	
total	114	9185.907		

$R^2 = 0.9667$

* : $p < 0.01$
** : $p < 0.05$
*** : $p < 0.001$

Table 1.9: 1998 normoxic treatments: Summary of the linear regression model of the effects of time in treatment, log treatment temperature, their interaction, number of eggs per vial, and replicates (r1-r4), on the time to 50 % of total hatching of *Hexagenia* embryos.

Regression

Variable	DF	Coeff.	SE	R^2
intercept	1	16.147	0.918	
time in treatment (time)	1	1.870***	0.069	0.88
log treatment temperature (temp)	1	-1.074**	0.329	0.05
temp x time	1	-0.405***	0.030	0.04
number eggs per vial	1	0.005	0.002	0.0
r1	1	1.584***	0.447	
r2	1	2.018***	0.462	0.0
r3	1	1.646***	0.473	
r4	1	0.767	0.442	

Analysis of Variance

	DF	SS	MS	F
model	8	9987.218	1248.402	606.151***
error	106	218.313	2.060	
total	114	10205.530		

$R^2 = 0.9786$

* : $p < 0.01$
** : $p < 0.05$
*** : $p < 0.001$

to time in treatment, therefore the time to hatching, after treament when placed in the normoxic 20 °C hatching conditions, was the same for all the embryos. In 1998, though the 28 d, 4 °C treatment eggs had the time to hatching increase by 1-2 d after treatment when they were placed in the normoxic 20°C hatching conditions. The 15 °C treatments (1998), unlike the lower temperatures, had a delay in hatching that was less than the time in treatment. There was also a slight delay in the 20 °C treatment vials compared to the control treatments in the petri dishes at 20 °C . After 15 days in treatment the delay in first hatching was approximately 3-4 d (Table 1.4 and 1.5)

Anoxia

Time in anoxia, at most temperatures, caused a delay in development that was equal to the time in treatment as indicated by the linear relationship between time to hatching and time in treatment (Figures 1.6b and 1.7b). In 1998, the increase in the delay in hatching was greater than the time in treatment for the eggs that were at 4 °C in anoxia for 28 d. After being in anoxia for the 28 d, the time to hatching at ambient conditions (20°C, normoxic water) the hatching was 1-2 d longer than normal (Table 1.5, Figure 1.7b)

The linear regression of the effects of time in treatment, log treatment temperature, their interaction, number of eggs per vial and the replicates on the time to hatching (both time to 7 % and time 50 % of total hatching) indicated that again time in treatment was the main significant effect ($p < 0.0001$) (Table 1.10 and 1.12 for 1997, and Table 1.11 and 1.13 for 1998).

Table 1.10: 1997 anoxic treatments: Summary of the linear regression model of the effects of time in treatment, log treatment temperature, their interaction, number of eggs per vial, and replicates (r1-r4), on the time to 7 % hatching of *Hexagenia* embryos.

Regression

Variable	DF	Coeff.	SE	R^2
intercept	1	14.324	0.352	
time in treatment (time)	1	1.084***	0.025	0.99
log treatment temperature (temp)	1	-0.203	0.140	0.0
temp x time	1	-0.317**	0.011	0.0
number eggs per vial	1	0.001	0.001	0.0
r1	1	-0.261	0.172	
r2	1	-0.268	0.173	0.0
r3	1	-0.028	0.171	
r4	1	-0.458	0.171	

Analysis of Variance

	DF	SS	MS	F
model	8	11693.112	1461.639	4156.200***
error	111	39.036	0.352	
total	119	11732.148		

$R^2 = 0.9967$

* : $p < 0.01$
** : $p < 0.05$
*** : $p < 0.001$

Table 1.11: 1998 normoxic data: Summary of the linear regression model of the effects of time in treatment, log treatment temperature, their interaction, number of eggs per vial, and replicates (r1-r4), on the time to 7 % hatching of *Hexagenia* embryos.

Regression

Variable	DF	Coeff.	SE	R^2
intercept	1	14.430	0.463	
time in treatment (time)	1	1.324 ***	0.026	0.98
log treatment temperature (temp)	1	-0.308	0.149	0.01
temp x time	1	-0.137***	0.011	0.01
number eggs per vial	1	-0.002	0.001	0.0
r1	1	1.074***	0.194	
r2	1	1.308***	0.196	0.0
r3	1	0.529*	0.203	
r4	1	0.589**	0.195	

Analysis of Variance

	DF	SS	MS	F
model	8	11317.020	1414.628	3252.358***
error	111	48.280	0.435	
total	119	11365.300		

$R^2 = 0.9958$

* : $p < 0.01$
** : $p < 0.05$
*** : $p < 0.001$

Table 1.12: 1997 anoxic treatments: Summary of the linear regression model of the effects of time in treatment, log treatment temperature, their interaction, number of eggs per vial, and replicates (r1-r4), on the time to 50 % of total hatching of *Hexagenia* embryos.

Regression

Variable	DF	Coeff.	SE	R^2
intercept	1	15.200	0.384	
time in treatment (time)	1	1.081 ***	0.0276	0.99
log treatment temperature (temp)	1	-0.038	0.152	0.0
temp x time	1	-0.033 **	0.0117	0.0
number eggs per vial	1	-0.003 **	0.001	0.0
r1	1	0.461	0.187	
r2	1	0.529	0.188	0.0
r3	1	0.277	0.187	
r4	1	-0.198	0.186	

Analysis of Variance

	DF	SS	MS	F
model	8	11472.210	1434.026	3438.761***
error	111	46.289	0.417	
total	119	11518.499		

$R^2 = 0.9960$

* : $p < 0.01$
** : $p < 0.05$
*** : $p < 0.001$

Table 1.13: 1998 anoxic treatments: Summary of the linear regression model of the effects of time in treatment, log treatment temperature, their interaction, number of eggs per vial, and replicates (r1-r4), on the time to 50% of total hatching of *Hexagenia* embryos.

Regression

Variable	DF	Coeff.	SE	R^2
intercept	1	16.132	0.623	
time in treatment (time)	1	1.388 ***	0.037	0.97
log treatment temperature (temp)	1	-0.449	0.202	0.01
temp x time	1	-0.153***	0.15	0.01
number eggs per vial	1	-0.001	0.002	0.0
r1	1	1.418***	0.261	
r2	1	1.617***	0.265	0.0
r3	1	1.221***	0.274	
r4	1	0.568	0.263	

Analysis of Variance

	DF	SS	MS	F
model	8	11968.596	1496.07	1892.923***
error	111	87.729	0.790	
total	119	12056.325		

$R^2 = 0.9927$

* : $p < 0.01$
** : $p < 0.05$
*** : $p < 0.001$

As in the normoxic treatments, there was a significant ($p < 0.05$ for 1997 and $p < 0.0001$ in 1998) time in treatment x log treatment temperature interaction (Tables 1.10-1.13).

Long term exposure to anoxia

After the eggs were in anoxia for 245 d (8 mo.) at 20 °C (1997 study), the time to 7% hatching was 16.8 ± 0.89 d and time to 50 % hatching of the total was 18.4 ± 0.55 d in ambient (20 °C, normoxic water) conditions. Both of these were delayed compared to the eggs, that were placed into ambient conditions immediately after being oviposited (Table 1.1). The time to first hatching was delayed by 1-2 d and the midpoint hatching was delayed approximately 3 d.

Discussion

Viability

There was a great difference in the overall viability of the *Hexagenia* embryos between 1997 and 1998. The viability (mean ± SD), in 1997, was atypically low, at 45.6 ± 9.2 % in the controls, compared to 80-95 % in other studies, including the 1998 study (Hunt 1953, Hanes 1992, personal observation). The reduced survivorship in 1997 was one of the reasons that the experiment was repeated in 1998.

The overall low viability in 1997 could be due to the cooler weather conditions of that spring and summer (personal observation). The eggs were collected after an unusually cold period in July and emergence of the adults was low which is why two nights were needed to collect enough eggs for the five replicates. It is noteworthy that the larval densities in western

Lake Erie were lower in 1998 (Ciborowski *et al.*, unpublished data). Schloesser (pers. comm.) attributes this to the failure of the 1997/1998 year class to hatch.

Another factor that could have caused the high mortality in 1997 was that the eggs were placed in treatment 2 d after they had been oviposited. From Chapter Two, this seems unlikely to be a factor since there was a greater viability in the mid (7 d old) and late (12 d old) embryos when exposed to anoxia.

The studies in both years showed that viability of the *Hexagenia* embryos declined with increased length of time in all the treatments for both 1997 and 1998 (Table 1.1, Figures 1.3 and 1.4). The regression models that best explained the decline in viability differed between the two years. In 1997, the main effects of oxygen, time in treatment, treatment temperature, their interaction and the covariates, replicate and number of eggs per vial, best explained log viability (Table 1.2). In 1998, treatment temperature and time in treatment were log transformed and used with the other independent variables to predict the effects on viability (Table 1.3). The difference in the regressions for the two years maybe a reflection of the fact that viability was lower in 1997 than in 1998.

In 1997, there was no significant difference among the temperature treatments for the anoxic conditions. In 1998, though, the survival was lower after being in anoxia at 4 °C for 28 days than at the other temperatures. Temperatures below 12 °C (Friesen *et al.* 1979) and 7 °C (Flattum 1963) have been shown to reduce the viability of *Hexagenia* eggs. Therefore, it is possible that 4 °C temperatures would decrease the viability even more under anoxic conditions. The difference in responses to the 4 °C temperatures between the two years could be due to the overall reduced viability of the 1997 eggs compared to the 1998 eggs.

Unexpectedly, after being in treatment for 28 days, the viability of the embryos from the normoxic treatment vials was less than those from the anoxic treatment vials in most cases (Table 1.1, Figures 1.3 and 1.4). When this happened in 1997, it was thought to be due to the high variability in the oxygen levels in the vials. Based on measurements taken at the end of treatment, the oxygen levels ranged from 0 - 9.1 mg/L in the normoxic treatment vials. The water in the vials was changed every 4 d, but this did not seem to be enough. Therefore, a pilot study was carried out to determine the oxygen consumption of the eggs in the vials (Appendix II). From the pilot study it was determined that the water had to be changed every 2 d to keep the oxygen levels above 6.0 mg/L. Even though this was done in 1998, and the oxygen levels ranged from 6.9-9.7 mg/L this did not improve the decline in the viability of the normoxic treatment eggs.

Long term exposure to anoxia

In 1997, longer term effects of anoxia were examined by keeping an extra set of eggs from each replicate in anoxia for 245 days (8 mo.). Even though the initial viability in 1997 was low there was still a mean ± SD percentage hatching of 16.4 ± 7.1 % after 8 mo. (Figure 1.5). Fremling (1967) studied the effects of reduced oxygen levels on *Hexagenia bilineata*. He found that at 12 °C in oxygen levels of 0.1-0.3 mg/L the eggs survived up to at least 380 days and in anaerobic conditions the eggs did not survive longer than 110 d. The lower viability in Fremling's (1967) second study could be accounted for by the water turning black and the presence of hydrogen sulfide gases (Fremling 1967).

Development

The anoxic treatments showed that the main factor that significantly ($p < 0.0001$) affected the time to hatching of the *Hexagenia* embryos was the length of time the embryos were in treatment (Tables 1.10-1.13). Anoxia delays the development at all temperatures. The amount of the delay in development was equal to the time in anoxia in most cases (Figures 1.6 and 1.7) indicating that no development was occurring in anoxic conditions. In 1998, the eggs in the anoxic conditions at 4 °C for 28 days had an increased delay in the time to hatch at 20 °C as did the eggs in the 28 day, normoxic, 4 °C treatments, as described below. Fremling (1967), also found an increase in the time to hatch at ambient (20°C, normoxic water) conditions after the *Hexagenia bilineata* eggs were held at oxygen levels of 0.1-0.3 mg/L for 110 days.

In normoxic conditions, log temperature was a significant ($p < 0.05$) factor in determining the time to development, unlike in the anoxic treatments where development did not occur when the eggs were in treatment. The development of the eggs was slower when they were in the colder treatments. There was a significant ($p < 0.0001$) log temperature x day interaction on the time to hatching, in that the longer the eggs were at the colder temperatures the longer they took to hatch (Table 1.6-1.9).

The results of the temperature effects are consistent with the literature (Flattum 1963, Fremling 1967, Friesen *et al.* 1979, Wright *et al.* 1982, Giberson and Roseberg 1992). Lower temperatures slow the rate of development (Flattum 1963, Friesen *et al.* 1979, Wright *et al.* 1982, Giberson and Roseberg 1992) as indicated by an increased time to first hatching and 50% of total hatching when the eggs are held at the lower temperatures (Figures 1.6 and 1.7). The almost linear relationship between time to first hatching and to 50% hatching and days in

treatment at 4.5 °C and 11 °C in 1997 and 5 °C and 9 °C in 1998 indicates that no development occurred while the eggs were in these temperature treatments. Development at 8 °C has been found to be slight in northern Manitoba population of *Hexagenia limbata* eggs (Giberson and Rosenberg 1992b), but there are records of no development at 10 °C (Hunt 1953, Heise *et al.* 1987) indicating the lower limit for development of the eggs is between 8 and 10 °C. Because the development is so slight at these temperatures, and the eggs in this study were only held at 8°C and 11 °C for a short time period (28 days) differences were not seen in the development compared to the 4 °C treatment.

After 14 and 28 days in the 5 °C treatment in 1998 there was a increase in developmental time when the eggs were incubated at 20 °C (Figure 1.7). This was not the case in 1997 where all the eggs at 4 °C had a developmental time equal to the normal time of 14 d at 20 °C. Flattum (1963), found that eggs stored at 7 °C, for different periods of time, and then brought up to 22 °C, would hatch in the normal incubation time period at 22 °C or in a longer incubation time period, but never shorter. The relationship between the days in refrigeration at 7 °C and the delay in incubation period was unpredictable (Flattum 1963). Delays in hatching have also been observed for other mayfly species (Humpesch 1960, Watanabe,1998).

Along with the reduced viability in the normoxic, 20 °C treatments, there was also a delay in the hatching time of the eggs with increased time in treatment compared to the eggs that were held at 20 °C in the petri dishes. This indicates that the environment in the sealed normoxic vials was different than the open petri dishes. Whether it was the variability in the oxygen levels or some other variable such as an accumulation of metabolic byproducts, the metabolism of the eggs was being interrupted within the vials.

Implications for the nymph populations in Lake Erie

As indicated in the introduction the ability of the *Hexagenia* embryos to survive over an anoxic event is important to the persistence of the population, especially, in a system like the western basin of Lake Erie. In the western basin, anoxia is intermittent and lasts 1-4 days when it occurs (Bartish 1987). It is long enough to decimate the *Hexagenia* nymph populations in the area of the anoxic event (Hunt 1953, Britt 1955a). When the nymph population is lost during a brief anoxic event (1-4 days) as in 1953, the presence and survival of eggs could allow for the recovery of the population once normoxic conditions return (Britt 1955a). The results of my study show that the *Hexagenia* eggs can survive periods of anoxia 1-4 d long without much of a reduction in their survivorship. The nymph population in western Lake Erie did recover the following year in the above case (Britt 1955b). The ability of the eggs to allow for the recovery of the population is only a small safeguard. Multiple occurrences of anoxia or the absence of eggs will cause the population to be lost, at least until conditions improve and recolonization begins.

The quality of the western basin of Lake Erie has improved greatly since the 1960s with the instigation of binational pollution abatement programs (Burns 1985, Makarewicz and Bertram 1991). The return of the *Hexagenia* population in the 1990s to the western basin is indicative of the water quality improvements (Reynoldson *et al.* 1989, Krieger *et al.* 1996). Anoxia is less likely to occur. It could still occasionally occur locally in some areas of the basin, either from stratification or from anoxic water spilling over from the central basin (Bartish 1984, 1987). The population of *Hexagenia* in the western basin has always been

variable (Reynoldson *et al.* 1989). The more resistant eggs could account for the continual recovery of the sensitive nymph population after a low population year.

Hexagenia eggs have been shown to withstand anoxia for at least 245 d in this study and 380 d in Fremling's (1967) study. This indicates the some eggs could survive long term burial in the sediment for at least up to a year. This could create a small egg bank. It would not be as extensive as some copepod species (De Stasio, Jr. 1989, Ban and Minoda 1992, Marcus and Lutz 1994), and *Daphnia* species (Carvahlho and Wolf 1989) which under go diapause and can last for years in the sediment.

The burial of the eggs in the anoxic sediments could also lead to the multiple cohorts found in the nymph population (Heise *et al.* 1987). If large groups of eggs become buried in the sediment and later resurface after a storm event, the subsequent hatching of these eggs could create a separate cohort.

Conclusion

In summary, this study has shown that *Hexagenia* eggs can survive periods of anoxia at temperatures ranging from 4 °C to 20 °C for up to 28 d with a 30 % reduction in its viability. The eggs will still hatch after 245 d in anoxia with further reduction in viability. While in anoxia, the development of the eggs essentially stops. Because *Hexagenia* nymphs are sensitive to low oxygen conditions (Hunt 1953) the ability of the eggs to survive anoxia can be important to the survival of the species if stochastic events of anoxia occur, such as in the western basin of Lake Erie.

Only early stage embryos were examined in this study. It is possible that different stages of the embryo will be affected more than others. The ability of embryos to survive at all stages of development is examined in the following chapter.

CHAPTER TWO: The effects of anoxia on *Hexagenia* at different stages of embryonic development

Introduction

In the previous chapter, the effects of anoxia at different temperatures on *Hexagenia* embryos were examined. The eggs used in that study were all embryos placed in treatment 1-2 d after being oviposited. Normally, the eggs are oviposited by female imagoes on the water's surface (Hunt 1953). The eggs then sink to the bottom sediments where they remain until hatching. The development of *Hexagenia* eggs is temperature dependent, but at 22 °C it takes about two weeks for them to hatch (Flattum 1963, Friesen *et al.* 1979, Hanes 1992). During that time, the *Hexagenia* eggs lying on the benthic sediments can be exposed to anoxia either through a stratification event or through burial (see General Introduction and Chapter One). Both of these events could occur at any point of embryological development.

The metabolic demands of an embryo will change as it develops. Salmon eggs were shown to have a higher metabolic demand in the later stages of embryonic development than in the early stage (Hayes *et al.* 1951). Having a higher metabolic demand may place the embryo under greater stress when exposed to anoxic conditions. A study on the effects of anoxia at different developmental stages of copepod eggs showed that newly spawned eggs were able to survive anoxia better than the later stages (Lutz. *et al.* 1994). Because there may be differences in the tolerance of the *Hexagenia* embryos to anoxic conditions, it is important to determine how anoxia will affect the different stages of embryonic development.

Early embryonic stage eggs can survive anoxia for at least 285 d at 20 °C (Chapter One). Since anoxic conditions can occur at any time, it is important to determine if the embryos not only survive anoxia at the early stages of development, but also at the later stages.

The main purpose of this study is to determine how anoxia affects the embryos at the different developmental stages. I examined the effects of anoxia on the development and viability on *Hexagenia* embryos at early, mid and late embryonic developmental stages. The effects of low temperature on developmental inhibition are greatest early in *Hexagenia* development, and the least at mid development (Friesen *et al.* 1979). Since anoxia also arrests the development of the eggs (Chapter One), anoxia may have the same effect on the different stages of development. On the other hand, as mentioned above, newly spawned copepod eggs were able to survive longer periods of anoxia than fully developed eggs (Lutz. *et al.* 1994).

I expect that the resistance of *Hexagenia* eggs to anoxia will vary among different embryonic stages. Embryos that are further developed will have their viability reduced more than newly released eggs. Fully developed eggs will possibly not survive, as the nymphs die within 30-48 h in anoxic conditions (Hunt 1953). I expect that the eggs that do survive the anoxic conditions will have their development delayed for the length of time the eggs are in anoxia, as in Chapter One.

Methods

Egg collection

Hexagenia eggs were collected from female imagoes at Colchester Harbour, Lake Erie, Ontario, on 28 June 1998. Five bags each containing eggs from 5-6 females were collected as outlined in the previous chapter. Eggs from groups of 5-6 females were used to ensure there would be enough eggs for each treatment. A few mg of dry potter's clay were added to the bags to prevent the eggs from sticking together, and to the glass of the Pasteur pipette when transferring the eggs to the scintillation vials.

Experimental design

Embryos were allowed to develop in aerated dechlorinated water, at 20 °C, to one of three stages based on Friesen's *et al.* (1979) categorization. At the first developmental (early stage) only the yolk is visible. Embryos reached the second (middle) stage when the embryo was discernible. The final (late) stage occurs when appendages and eyespots are visible (Friesen *et al.* 1979). At 20 °C, newly oviposited *Hexagenia* eggs take approximately 13-14 d to develop and hatch (Chapter One). Based on this and daily monitoring of the embryos, the embryos reached the early, middle and late stages at days 1, 7 and 12, respectively.

The early (1 d old), middle (7 d old) and late (12 d old) eggs were transferred to anoxic conditions on June 29, July 6 and July 11, respectively. The eggs remained in anoxic conditions for intervals of 0, 1, 4, 7, 14 or 28 days.

After their exposure to anoxia the embryos were transferred back ambient conditions, in aerated, dechlorinated water at 20 °C, to allow for hatching. The eggs were monitored daily for hatched nymphs which were counted and removed.

The experimental design is summarized in Figure 2.1. There were five replicates per treatment. One set of eggs remained in aerated dechlorinated water to determine the hatching success percent and development time (d) in normoxic conditions.

Experimental procedure

This experiment was carried out for its entirety in a walk-in environmental chamber set at 20 °C with a photoperiod of 16 h light and 8 h dark.

Development conditions

Once the eggs were collected they were stored at 20 °C in the dechlorinated, aerated water that they were collected in. Eggs for the early development stage were placed into treatment the day after collection. From the remaining eggs in the replicates, a few eggs were randomly removed from the bags to monitor the development of the embryos. Eggs were examined 5, 6 and 7 d after collection for the mid developmental stage, and 10, 11, and 12 d after collection to determine the start of the late stage. The egg shells were clear enough to allow for observation of the embryos under 40x magnification of a dissecting microscope. When about 50 % of all the eggs were at the

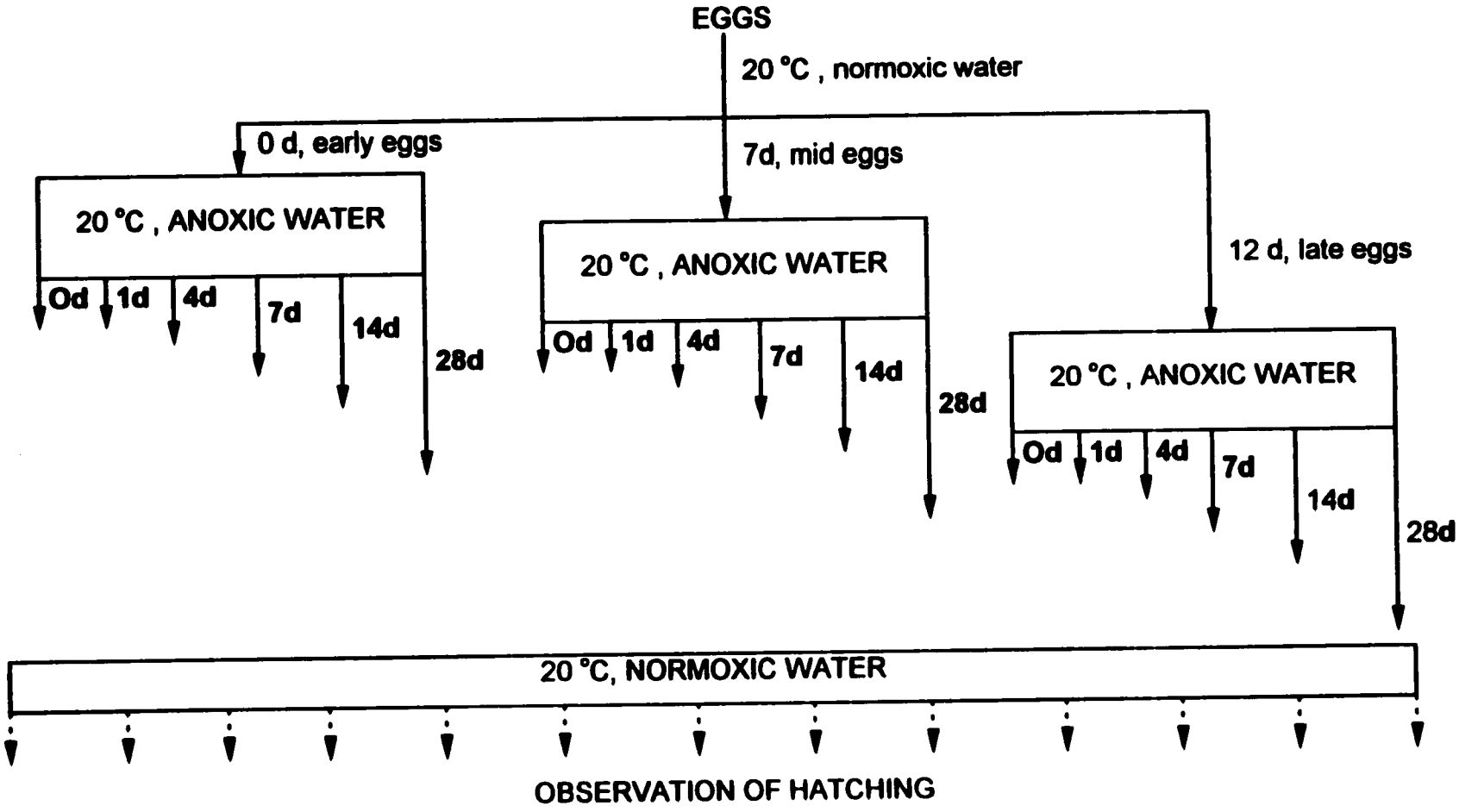


Figure 2.1: ***Hexagenia*** **egg hatching treatments**

desired development stage, eggs were taken from each replicate bag and placed into treatment at the same time.

Treatment Conditions

Once the eggs were at the early, middle or late developmental stages, they were transferred to 20 mL scintillation vials. A Pasteur pipette was used to remove an aliquot of water and eggs from the bottom of a collection bag. The eggs were allowed to settle at the tip of the pipette, and a drop of eggs was placed at the bottom of the scintillation vial. One drop contained (mean ± SD, n = 75) 259 ±90.5 eggs. Once a drop of eggs had been placed into a vial, the vial was filled with anoxic, carbon filtered dechlorinated water. The water was made anoxic by bubbling it with 99.95% nitrogen gas. The vials were filled so that the water was just over the top of the vial (to eliminate air bubbles in the vial), immediately capped, and then sealed with Parafilm.

The eggs remained in anoxic conditions for 0, 1, 4, 7, 14 and 28 days. The embryos were then transferred 60 mm diameter x 15 mm deep plastic petri dishes containing aerated dechlorinated water.

Transfer of eggs to hatching conditions

Before placing the embryos into aerated dechlorinated water, for hatching, the oxygen concentration of the water in the vials was measured using a Clark style O_2 microelectrode (Diamond General Inc., Ann Arbor, MI), immediately after uncapping, to ensure that anoxia had been maintained. The oxygen meter was calibrated using aerated dechlorinated water

(representing 100% O_2 saturation) and nitrogen-deoxygenated dechlorinated water (0% O_2 saturation).

The anoxic water was then carefully pipetted from the vial, leaving the eggs behind in approximately 2 mL of water . The eggs were then washed, using aerated dechlorinated water, into a plastic petri dish. The petri dishes were filled with aerated dechlorinated water and the eggs remained in the dishes until they till they hatched.

Monitoring for hatching

The eggs in the petri dishes were monitored daily for hatching, under a dissecting microscope. Hatched nymphs were counted and removed. The daily counts of nymphs were used to determine time to first (7%) hatching and mid-point (time to 50 % of total hatched) hatching, and the percentage hatching. I monitored the eggs until no further hatching was likely to occur, as indicated by the eggs browning or deteriorating internally (Friesen *et al.* 1979).

Statistical analysis

The effects of the stage of embryonic development, time in anoxia, replicate and their interactions on development (time to 7% hatching and 50% of total hatching), and viability were analyzed by analysis of variance using the general linear model procedure (SAS Institute Inc. 1985).

Results

Oxygen measurements using the Clark style O_2 microelectrode (Diamond General Inc., Ann Arbor, MI) confirmed that all the treatment vials were anoxic (<0.1 mg/L). Some of the 0 d treatment vials had water with oxygen measurement as high as 1.0 mg/L, but all the 1 d treatment vials were anoxic. Therefore, I was assumed that all vials became anoxic within 1 d.

Viability

Hexagenia embryos at early, mid and late embryonic stages were able to survive periods of anoxia for at least 28 d, with a relatively high viability (Figure 2.2). The viability of the early stage embryos, that were placed in anoxia for 1, 4 and 7 d, was approximately 10 percentage points lower (70-76%) than the mid and late stage embryos (85-88%) that were in anoxia for the same lengths of time (Figure 2.2). The mid developmental stage embryos exhibited a decline in viability (mean ± SE) from 85.3 ± 3.3% in the control embryos to 67.2 ± 2.5 % in the eggs that were exposed to anoxia for 28 days. The decline was first evident after 7 d exposure to anoxia (Figure 2.2). The viability of the late stage embryos stayed relatively stable, and high across all the periods of anoxia; the late stage embryos started with an 88.5 ± 4.1 % viability (mean ± SE) in the control treatments and after being in anoxia for 28 d the viability was 83.2 ± 3.5 %.

The influence of the three variables (replication, developmental stage and days in anoxia) on the viability was analyzed by 3-way ANOVA for their influence on the viability (Table 2.1). The developmental stage, the replication, and days in anoxia all had highly

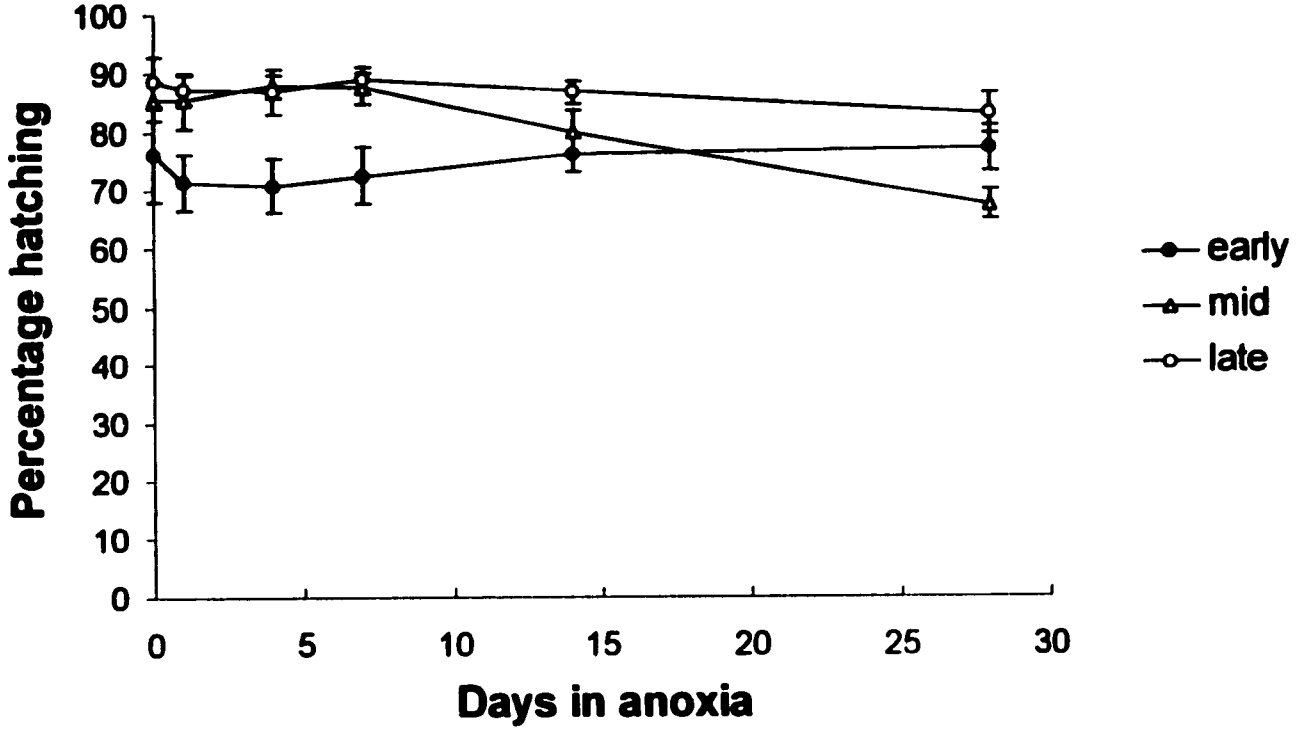


Figure 2.2: Viability (mean ± S.D %, n=5) of *Hexagenia* embryos after being in anoxic conditions for 0-28 days at early, mid, and late embryonic stages

Table 2.1: Summary of three-way ANOVA of the effects of replication, development stage, days in anoxia and their interactions on the viability of *Hexagenia* eggs.

Variable	df	Sum of squares	Mean Square	F Value
replication (rep)	4	2151.489	537.872	15.96***
development stage (stg)	2	2725.039	1362.519	40.42***
rep x stg	8	428.254	53.532	1.59
days in anoxia (day)	1	449.897	449.897	13.35**
day x rep	4	183.559	45.890	1.36
day x stg	2	1043.340	521.670	15.48***
day x rep x stg	8	240.919	30.115	0.89
error	60	2022.528	33.709	

* : $p < 0.05$
** : $p < 0.001$
*** : $p < 0.0001$

significant ($p < 0.001$) effects on the viability of the embryos, as did the interaction between the developmental stage and days in anoxia.

Overall, the viability varied significantly ($p < 0.001$; 3-way ANOVA, see Table 2.1) among the replicates (Figure 2.3).

Development

Exposure to anoxia affected the time to hatching of the embryos at all three stages of development. The relationship between time to hatching (both time to first hatch and 50 % of total) for the different treatments was linear in relation to the days that the embryos were in treatment at all stages (Figure 2.4). Time to 50 % of total hatch to occur at 20 °C in normoxic water was around 15 days for the embryos in all treatments (Figure 2.5). No hatching occurred while the eggs were in the anoxic conditions.

The 3-way ANOVAs showed that both time to 7 % hatching and to 50 % of the total was significantly ($p < 0.0001$) affected by the time the embryos spent in anoxia (Table 2.2 and Table 2.3). The developmental stage was only marginally significant ($p < 0.05$) for time to 50 % of the total hatch. The interaction of the developmental stage x time in anoxia was marginally significant ($p < 0.05$) for the time to 7 % hatching. Replication was highly significant ($p < 0.001$) in both cases. The interaction of replication x stage was significant ($p < 0.001$) for time to 50 % of total, but not time to 7% hatching.

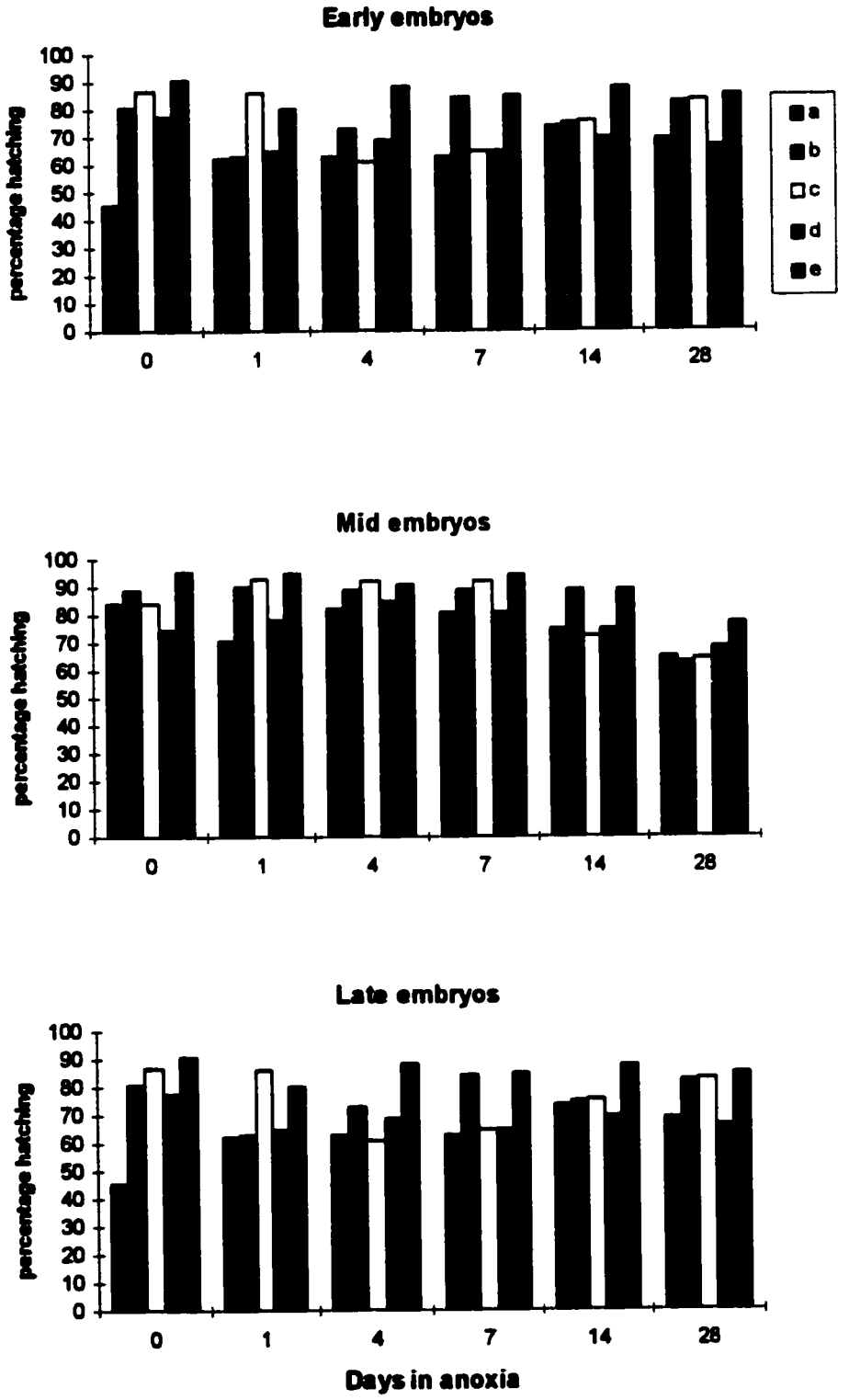


Figure 2.3: Viability (percentage hatching) of the five replications (a-e) of *Hexagenia* embryos that were in anoxic conditions for 0-28 days at early, mid and late stages of development.

Table 2.2: Summary of three-way ANOVA of the effects of replication, developmental stage, days in anoxia and their interactions on days to 7% hatching in *Hexagenia* embryos.

Variable	df	Sum of squares	Mean Square	F Value
replication (rep)	4	4.102	1.025	7.26***
development stage (stg)	2	0.652	0.326	2.13
rep x stg	8	0.997	0.125	0.88
days in anoxia (day)	1	8424.01	8424.017	59605.57***
day x rep	4	1.254	0.314	2.22
day x stg	2	1.009	0.505	3.57*
day x rep x stg	8	0.573	0.072	0.51
error	60	8.480	0.141	

* : $p < 0.05$
** : $p < 0.001$
*** : $p < 0.0001$

Table 2.3: Summary of three-way ANOVA of the effects of replication, developmental stage, days in anoxia and their interactions on 50 % of total hatching in *Hexagenia* embryos.

Variable	df	Sum of squares	Mean Square	F Value
replication (rep)	4	10.496	2.624	14.25***
development stage (stg)	2	1.547	0.773	4.20*
rep x stg	8	6.563	0.82.	4.45**
days in anoxia (day)	1	8382.010	8382.010	45515.63***
day x rep	4	1.910	0.476	2.59*
day x stg	2	0.334	0.167	0.91
day x rep x stg	8	1.530	0.191	1.04
error	60	11.049	0.184	

* : $p < 0.05$
** : $p < 0.001$
*** : $p < 0.0001$

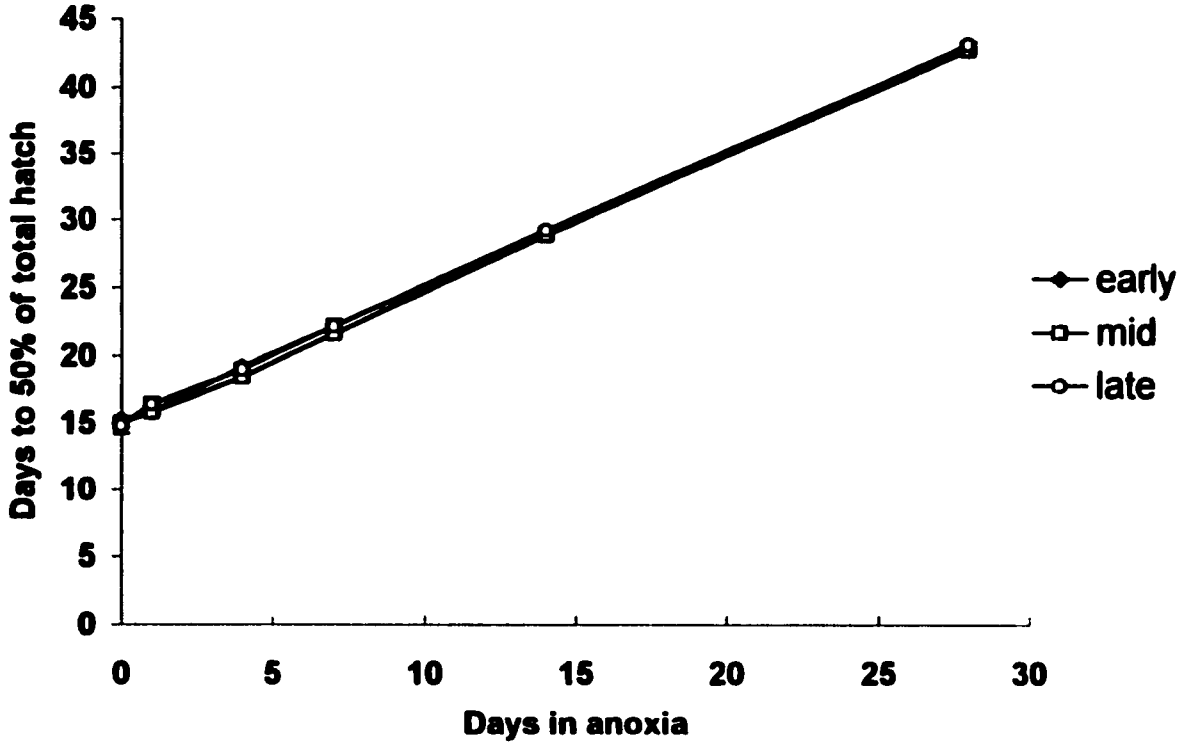


Figure 2.4: Time to 50 % of total hatching (± S.E.) of *Hexagenia* embryos after being placed in anoxic conditions for 0, 1, 4, 7, 14, and 28 days at early, mid, and late stages of development.

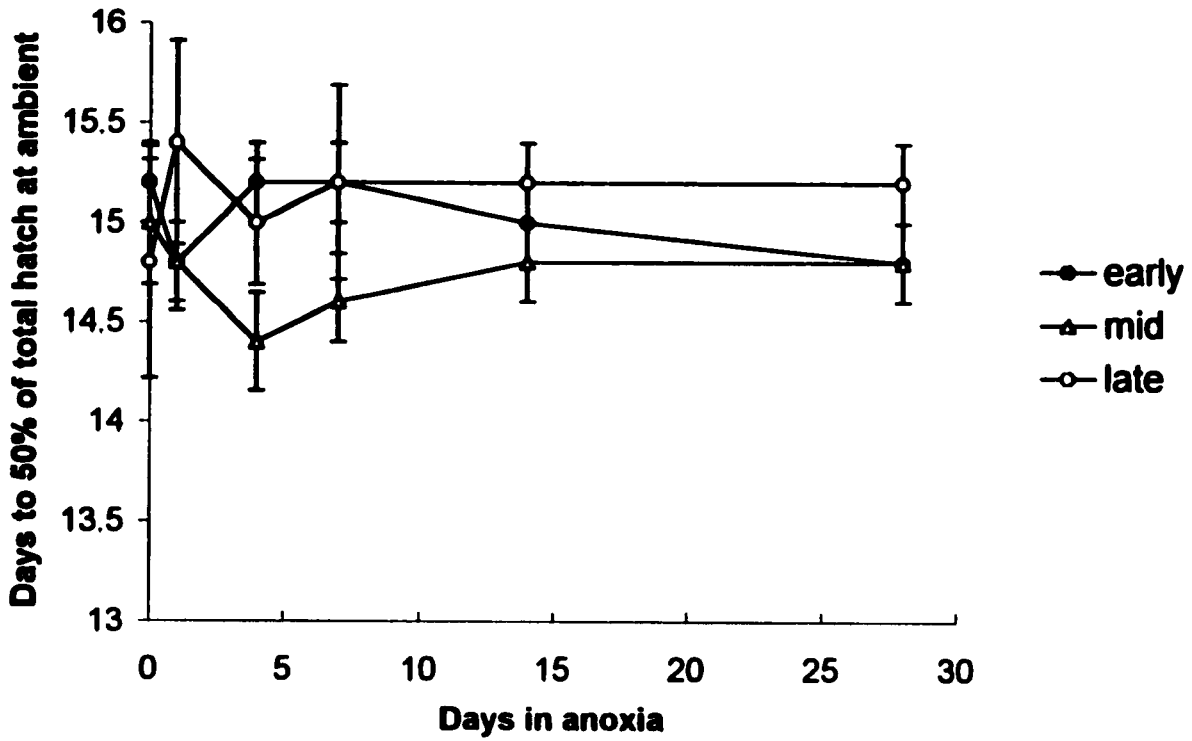


Figure 2.5: Mean (± S.E) days to 50% of total hatch of *Hexagenia* embryos in the hatching conditions of normoxic water at 20 °C where the eggs were placed in anoxic conditions for 0 to 28 days at early, mid and late stages of development.

Discussion

Viability

Viability remained relatively high for the *Hexagenia* embryos at all three (early, mid and late) developmental stages for the different exposure periods to anoxia. The main effect on the viability when the embryos were in anoxia was the developmental stage (Table 2.1). The effect of anoxia, over time on the viability of the embryos was different at the different embryonic stages (Figure 2.2).

The viability of the early stage embryos stayed relatively stable after the different periods of exposure to anoxia (~ 76 % at 0 d exposure eggs to ~77 % after the 28 d exposure) (Figure 2.2). In Chapter One, by contrast, when the early stage embryos were exposed to anoxia at 20 °C for the same time periods, the viability decreased with the longer exposure times. There was a decrease in the viability of the embryos from the control eggs (75.5 ± 4.1 %) to the 28 d exposure eggs (63.8 ± 3.1 %) (1998 data, Chapter One). A possible reason for the discrepancy between the two studies is the greater amount of handling of the eggs that occurred in the Chapter One study. In Chapter One, 50 % of the anoxic water was replaced in the treatment vials every 2 d , but in this study the vials remained sealed while the eggs were in treatment.

The viability of the late (~ 88%) and mid (~85-87%) stage eggs was higher after the short term (1-7 d) exposure treatments to anoxia compared to the embryos that were exposed to anoxia for the same time period at the early stage of development (~ 70-76%).

The viability of the mid stage embryos was affected the most by long term anoxia. The viability (mean ± SD) was initially 85.3 ± 3.3 % in the control treatments, but it started to decrease after 14 d, and was at 67.2 ± 2.5 % after 28 d of anoxia (Figure 2.2).

For the late stage eggs, the viability remained high between the short term and longer term exposures to anoxia. The initial viability of the late stage eggs was 88.5 ± 4.1% and the 28 d eggs had a viability of 83.2 ± 3.5 %. These were the highest viability of the three developmental stages.

It is noteworthy that the late stage eggs were the least affected by the different exposures to anoxia. It contradicts my initial assumption that the late stage eggs would be the most effected by anoxic conditions, as in the copepod eggs (Lutz. *et al.* 1994). In this study, the mid stage eggs were most affected by anoxia, yet low temperatures (4 °C), which also inhibit development of the eggs, had the least effect on the mid stage eggs (Friesen *et al.* 1979).

Another factor that affected the viability of the eggs at all stages was the replication. Each replicate consisted of eggs pooled from 5-6 female imagoes. Some replicates showed high viability consistently while others always had a low viability (Figure 2.3). It is possible that the difference in viability between the replicates reflects the differences in the ability of the eggs from the same female to tolerate stress. Hanes (1992) found that there was a maternal effect on the survivorship of the larvae.

Development

The development of *Hexagenia* embryos was affected by anoxia at all embryonic stages. The longer the eggs were in anoxia the longer the time to hatch (Figure 2.4). The linear relationship between time to hatching and days in anoxia, for the early, mid and late developmental stages, indicated that no development occurred while the eggs were in anoxia. This is also shown in Figure 2.4 where the time to hatch at 20 °C in normoxic conditions (ambient) stays around 15 d for all treatments.

The ANOVAs for time to first hatching and to 50% of total hatch showed that time in anoxia was the factor that most strongly affected the development (Table 2.3).

Replicate was also significant, but to a lesser degree. This could be due to slight differences in the metabolic rates of eggs from different females as each replication was made up of eggs from 5-6 different females.

There was a slight effect of developmental stage on the time to hatching. The effect was marginally significant for time to 50% of total hatch and was significant in its interaction with day for time to 7 % hatching. On average, the difference between the time to hatching among the different stages was no more than 1 d (Figure 2.5).

Conclusion

This study revealed that *Hexagenia* embryos, at all stages of development, from after they have been oviposited by a female imago until at least one day before hatch can tolerate anoxic conditions for up to at least 28 days. This would allow eggs that experience anoxia to survive over a short term period. The eggs will likely tolerate longer

periods of anoxia. Early stage embryos have been shown to be viable after being held in anoxic conditions for 245 d (Chapter One) and 380 d (Fremling 1967). Friesen *et al.* (1979) found that eggs at early to late stages of development were able to tolerate cold temperatures that also inhibited development for 365 d with reduced viability. The ability of the eggs to tolerate the anoxic events is probably due to their lack of development during anoxia.

The ability of *Hexagenia* embryos to withstand anoxia at all developmental stages is an advantage to the *Hexagenia* nymph populations in lakes and large rivers where they occur (Hunt 1953). Short term anoxic events can occur in summer due to intermittent stratification, such as in the western basin of Lake Erie (Bartish 1984). An anoxic event will cause the nymph population to crash (Britt 1955a) due to their low tolerance of oxygen below 1.0 mg/L (Hunt 1953). Once the anoxic event passes any eggs present can hatch and repopulate the area with nymphs. This will allow the *Hexagenia* population to persist if anoxic events occur infrequently.

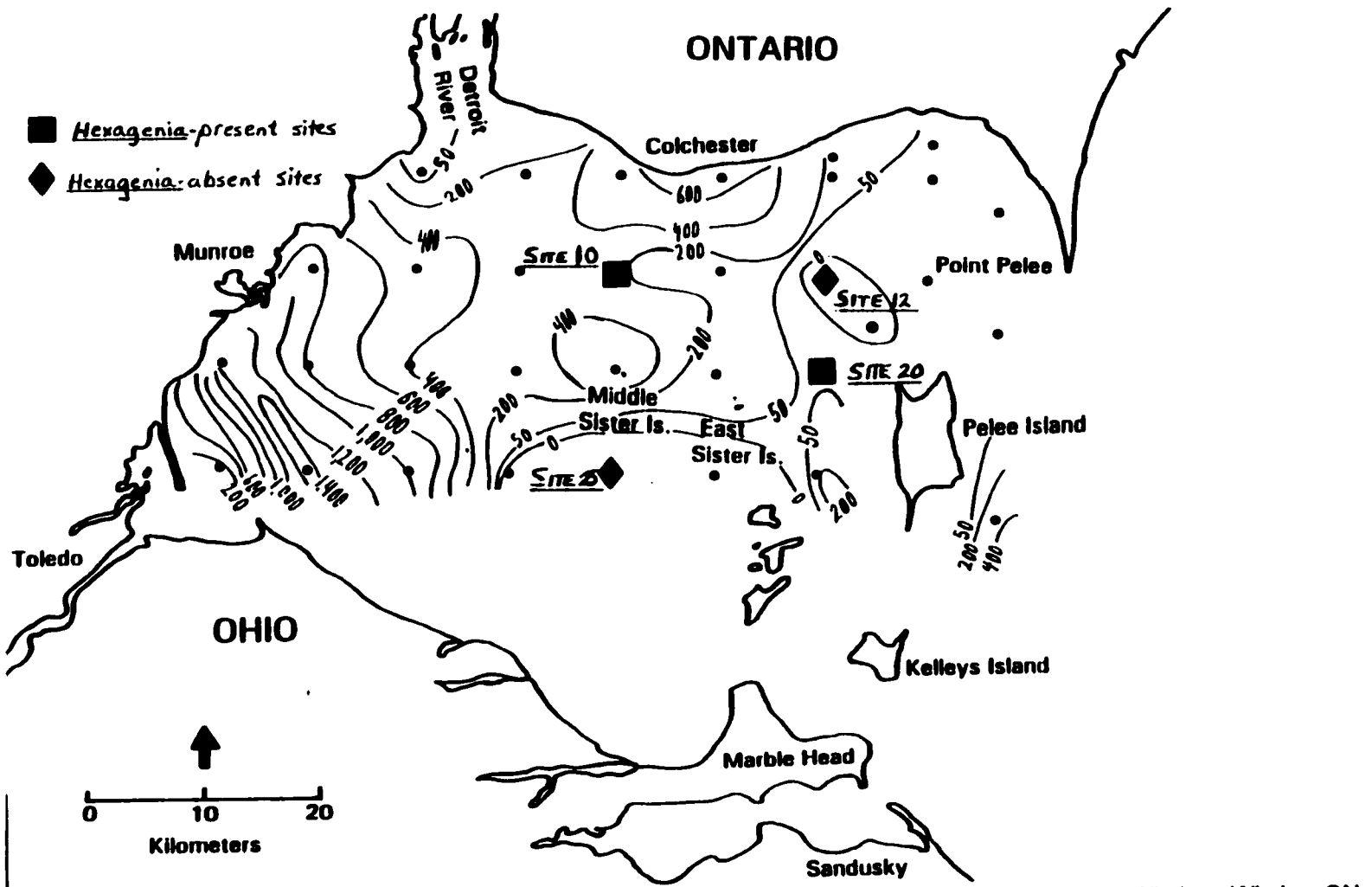
In terms of long term survival, *Hexagenia* eggs on the lake bottom can become buried within the anoxic layers of the sediment, 2-2.5 mm below the sediment surface (Gundersen and Jorgensen 1990, Ventling-Schwank and Livingstone 1994). Burial could expose a *Hexagenia* egg to a long term anoxic event at any time in its developmental stage. Survival of the egg during this period would allow it to hatch once it resurfaces to normoxic conditions. Different re-emergent times of eggs from the sediment in may partially explain the multiple cohorts found in the nymph population (Heise *et al.* 1987).

CHAPTER THREE: The effects of hypoxia on *Hexagenia* embryos in sediment cores from western Lake Erie and implications of egg burial by nymph bioturbation

Introduction

Hexagenia populations have been reappearing in the western basin of Lake Erie since the early 1990s (Krieger *et al.* 1996, Schloesser *et al.* in review), after their disappearance in the 1960s (Carr and Hiltunen 1965, Britt *et al.* 1973). By 1996, *Hexagenia* nymphs still had not recovered in some areas of the western basin (J.J H. Ciborowski, University of Windsor, Windsor, ON). One area was in the Pigeon Bay region, west of Point Pelee, and the other in an area south of Middle Sister Is. (Figure 3.1). It is uncertain why *Hexagenia* nymphshave not reappeared in these areas when they have returned to the surrounding areas.

The loss of *Hexagenia* in the western basin was attributed to increased occurrences of anoxic events with the eutrophication of the basin (Britt *et al.* 1973). It is also possible that contamination of the sediments with toxins and metals could have played a role in the disappearance of *Hexagenia* (Burns 1985). Alternatively, the areas may be too far away from established populations to have been reached by an adequate number of ovipositing adults. With the incorporation of pollution abatement programs in the 1970s (Burns 1985) and the establishment of zebra mussels (*Dreissena polymorpha*) in 1989 (Griffith *et al.* 1991) the water quality of western Lake Erie has improved enough to allow the re-establishment of



map of *Hexagenia* densities courtesy of J.J.H. Ciborowski, University of Windsor, Windsor ON.

Figure 3.1: Western Lake Erie core sampling sites mapped with *Hexagenia* nymph distributions (mean number/m^2, based on n=5 Petite ponars) for May 1997.

Hexagenia nymphs (Krieger *et al.* 1996, Schloesser *et al.* in review).

The absence of *Hexagenia* in the two areas referred to above may reflect the fact that water quality has not improved enough in these areas to allow for colonization of *Hexagenia*. *Hexagenia* are considered to be biological indicators reflecting the return of a lake to mesotrophic conditions from eutrophic conditions (Reynoldson *et al.* 1989).

The *Hexagenia*-absent areas, above, based on the sediment and the depth, seem to be suitable for the re-establishment of nymphs. The sediment in these areas is mud silt (pers. observ.), the preferred sediment type of *Hexagenia* nymphs for colonization (Wright and Mattice 1981). The depth at these sites (11.5 m) should not be a factor as nymphs have been found as deep as 17.5 m (Neave 1932). If it is not habitat constraints, then it is possible that the environmental conditions, in terms of epibenthic oxygen conditions or contaminants in the sediment are not suitable for *Hexagenia*.

Incidences of periodic epibenthic anoxia could still be occurring in these *Hexagenia*-absent areas. The biological oxygen demand of the sediments in these areas may still be high enough to allow for anoxia to occur during a stratification event (Bartish 1984). Bartish (1984) recorded some anoxic events in the Pigeon Bay region and around the region south east of Middle Is. between 1980 and 1981. For the Pigeon Bay region not only could episodes of anoxia occur from a stratification event, but spillover of anoxic water from the central basin may also create anoxic conditions (Bartish 1984, 1987).

Hypoxia can affect both the nymphs and eggs of *Hexagenia*. Nymphs will die within 48 h when exposed to oxygen levels below 1.0 mg O_2 /L (Hunt 1953). Chronic hypoxia, has been shown to reduce the survival of nymphs, with increased effects at

higher temperature (Winter *et al.* 1996). Only 20% of *Hexagenia* nymphs survived after three weeks of exposure to 0.82-0.84 mg/L at 14 °C (Winter *et al.* 1996). Survival of the embryos also decreases, but at a much slower rate, when exposed to anoxia (Fremling 1963, Chapters One and Two). After 3 mo. in anoxia the survival of *Hexagenia* was 16% for newly laid eggs (Chapter One). Of the embryos that survive anoxia the development is delayed for at least the length of the exposure to anoxia (Chapters One and Two). Based on this, short term exposure to anoxia from stratification (4 days (Bartish 1984)) would probably eradicate the nymphs in the area but the eggs should be able to survive anoxia albeit with delayed hatching.

Contaminants in the sediment could also prevent the establishment of *Hexagenia* populations by affecting both the embryos and nymphs. *Hexagenia* nymphs are sensitive to sediment contaminants and are used in bioassays to evaluate sediment toxicity (Ciborowski *et al.* 1992). The presence of oil in the sediments will also inhibit the presence of nymphs in sediments (Hiltunen and Schloesser 1983, Schloesser *et al.* 1991). The embryos are also sensitive to contaminants in the sediment and have been used in toxicity testing (Friesen 1979). Metholxychlor, saline ground water and cadmium have all completely suppressed hatching in *Hexagenia* eggs (Friesen 1979).

Another possibility that would prevent the return of *Hexagenia* to these sites, besides habitat quality, is lack of colonization. It is possible that females are not depositing eggs at these sites, or currents are carrying the eggs away from these areas. An absence of eggs would mean that there would be no *Hexagenia* nymphs, unless nymphs migrate in from other areas. If eggs are present at these sites and hatching then it may be

possible that the nymphs are migrating out of the areas because of unsuitable habitat (Hunt 1953) or the nymphs are dying.

These are some possible reasons as to why *Hexagenia* are not present at these sites. Other ecological factors could also be involved, such as resource competition with other species, or high predation.

This study was done to examine why there are no *Hexagenia* nymphs in the western basin in the middle of the Pigeon Bay and in the area S. of Middle Sister Is. from the perspective of the egg stage. Three possibilities were looked at. One was to determine if *Hexagenia* embryos would hatch in the sediment from the two areas where there were no *Hexagenia* nymphs. If contaminants are present in the sediment they could prevent the eggs from hatching (Friesen 1979). The second possibility to be examined was the effects of short term anoxia (4 days). From studies prior to this one it is known that embryo development will be delayed when exposed to anoxia, but the embryos should hatch once oxygen levels rise again (Fremling 1969 and see Appendix I).

The final possibility was that there are no eggs reaching the *Hexagenia*-absent areas. If there are no eggs being deposited by female imagoes or being carried by currents and sedimenting out of the water column in these areas then there will be no colonization, unless nymphs swim in and settle.

The first and third objective of this experiment were evaluated. There were problems setting up an anoxic environment for the second objective. Instead the effects of hypoxia ($O_2 < 6.5$ mg/L) on the hatching of eggs were examined. Besides these

objectives, interesting observations on the effects of large nymphs on the eggs were also observed.

Methods

Sediment cores were hand collected from four sites in western Lake Erie (Figure 3.1). The cores were kept intact and used in the laboratory in an attempt to examine the effects of anoxia on *Hexagenia* eggs in a more natural environment than in the previous two chapters.

Sites

The sites in the western basin of Lake Erie (Figure 3.1) were chosen based on the 1996-1997 *Hexagenia* nymph distribution data collected by Ciborowski *et al.* (1997); two sites (10 and 20) had *Hexagenia* nymphs present and two sites (12 and 25) had no nymphs present (Schloesser *et al.* in review). All sites had muddy silt sediment and were 11-12 m deep (Table 3.1).

Experimental design

To examine the hatchability of *Hexagenia* eggs in the western basin of Lake Erie sediment cores were collected from the four areas listed above in the basin: two areas where *Hexagenia* nymphs were present and two where they were absent. The cores from each site had 50 *Hexagenia* eggs added and were then placed in one of two oxygen

Table 3.1: Summary of core site locations and characteristics. Nymph densities (mean ± SD) based on petite ponar sampling (n=5) in May 1996 and 1997 (J.J.H Ciborowski, University of Windsor, Windsor, ON)

				Nymph density (nymphs/m^2)	
Site	Location		Depth (m)	1996	1997
10	41°54'00 N	82°58'04 W	11.6	44.4 ± 51.32	213.3 ± 115.04
12	41°54'00 N	82°46'00 W	11.6	0.0	0.0
20	41°49'02 N	82°46'00 W	11.6	8.9 ± 19.88	35.6 ± 37.18
25	41°44'02 N	82°58'04 W	11.0	0.0	0.0

treatments (n = 5). The first treatment was continually aerated to keep oxygen levels at 100% saturation. The second treatment was to be allowed to go anoxic for 4 days. As explained further on, this did not occur, but the cores did become hypoxic (<6.5 mg O_2 /L water). After an incubation time sufficient to allow hatching, the eggs and any hatched nymphs were recovered from the top 2 mm of sediment in the cores to determine the percentage hatching. Each treatment had five replicates from each site.

These treatments were set up to determine if the absence of *Hexagenia* nymphs in some areas of the western basin could be due to the prevention of the eggs hatching because of sediment quality or occurrences of anoxic events.

Additionally, five extra cores were collected from each site to determine if eggs were present at the sites when the cores were obtained.

Field Collection of the Sediment Cores

From each of the four sites, 15 cores were collected. Cores at sites 10 and 12 were collected 28 May, 1997 by Domenic Paoneossa (University of Windsor, Windsor, ON). These cores were hand-collected from a 1 m^2 box corer collected by the Environment Canada Research Vessel *Limnos*. Cores at sites 25 and 20 were hand-collected by SCUBA by Jocelyn Gerlofsma and Bob Wickett (University of Windsor, Windsor, ON) on 10 June 1997. The cores were obtained before the first emergence of *Hexagenia* adults (L.D. Corkum and D. Laing, University of Windsor, Windsor ,ON, pers. comm.).

The cores were collected using polycarbonate tubes measuring 6.5 cm diameter x 30 cm long. The tube was pushed 1/2-3/4 of the way down into the sediment and topped up with lake water. The tube was then capped on the top, lifted out and capped on the bottom. Duct tape was used to seal the caps in place. The cores were stored at 4-5 °C in a cold room until they were to be used in the laboratory.

To keep the volume of sediment versus the volume of water in each tube consistent, sediment had to be removed from some of the tubes in the laboratory. To remove the sediment without disturbing the top layers, the bottom cap of the tube was carefully removed. Once air was let at the top, by slowly lifting the top cap, the sediment slid down, in an intact column, out of the bottom. When the top of the sediment reached the halfway point of the tube, a flat plate was slid across the bottom, stopping the flow of sediment. The top cap was pushed back on so that the bottom could be recapped. The top halves of the cores were filled with water collected in Lake Erie from Kingsville, Ontario.

Laboratory environmental conditions

The cores were transferred to a 20 °C environmental chamber on 12 June 1997. They were placed under fluorescent lighting set on a 16 h light/ 8 h dark cycle. The sides of the tubes were covered with black Bristol board and had the top edge blacked out with black permanent markers to prevent light coming in from the sides of the tube. The tubes were aerated using syringe needles and capillary tubing (Hanes and Corkum 1989). The tubes were aerated and allowed to equilibrate in these conditions for 14 d before the eggs were added.

Collection of eggs

A bag containing eggs oviposited by 27 female *Hexagenia* imagoes was collected by L. D. Corkum (University of Windsor, Windsor, ON) at the Colchester Harbour, Lake Erie, Ontario on 25 June 1997. Female imagoes were collected in the evening by the light in the harbour. The female imagoes were grasped by the wings and placed on the surface of aerated dechlorinated water in a soil bag. Once placed on the water the female released her eggs into the water. The next day the females were removed and placed into 70% ethanol.

Treatments

Five cores were randomly selected from each of the four sites and placed in one of three treatments. One set of cores was continually aerated and had 50 *Hexagenia* eggs/core added. The second set, also with 50 eggs/core added, was made hypoxic 4 d after the eggs were added until the end of the experiment. The last set of cores was aerated, but no eggs were added.

For the tubes to which eggs were added, the eggs were counted out and placed in a petri dish where they were recounted. To add the eggs to the tube, the petri dish was rinsed, using Lake Erie water, into the tube.

After the eggs were added, aeration continued in all cores for 4 d. On the fourth day the set of cores that were to be made hypoxic were continuously bubbled with 99.95 % nitrogen gas, instead of atmospheric air. These cores were to be made anoxic but the

nitrogen gas ran out after 24 h preventing the cores from becoming anoxic. These cores did become and remained hypoxic until the removal of the eggs. At 18 h after bubbling with nitrogen gas the oxygen concentration was approximately 3.0 mg/L. At this time, the tubes were covered with plastic to prevent atmospheric air from entering cores. The oxygen concentrations did rise in the hypoxic cores after the nitrogen gas ran out but the did not exceed 6.5 mg/L.

Aeration was continued in the other two sets of tubes and at 18 h the oxygen concentration was 8.6 mg/L. The oxygen concentrations in the tubes were measured using an Clark style O_2 microelectrode (Diamond General Inc., Ann Arbor, MI). Oxygen levels were measured 3 more times during the course of the experiment.

Removal of eggs from the cores

The eggs were to be removed from the cores after the period of peak hatching, four days after first larvae hatch (pers. observ.). Monitoring of the peak hatching was done using a subsample of the eggs, collected for the cores, placed in 9 petri dishes (6 cm diameter x 15 mm high). These eggs were kept beside the cores and therefore exposed to the same temperature and light conditions. The dishes were filled with lake water, which was continuously aerated using capillary tubing and syringes (Corkum and Hanes 1989). The eggs were easily monitored daily for hatching under a dissecting microscope at 40x magnification.

On the fourth day of hatching, the eggs and nymphs in the petri dishes were placed in 20 mL scintillation vials with 70% ethanol so they could be counted later to determine the percentage hatching. At this time the cores were taken down.

To recover the eggs and nymphs from the cores, the top 2 mm of sediment were removed from the tube. First, part of the water was poured off gently through a 90 μm mesh sieve. When the sediment started to be disturbed by the pouring off of the water, the rest of the water was removed using a pipette. Once the water was removed, a cap was placed on top of the tube so that the cap on the bottom could be removed without the sediment falling out. A 6 cm diameter PVC cap that fit into the tube was used to push the sediment from below up to the top of the tube. Once the surface of the sediment reached the top, 2 mm was marked above the bottom of the sediment. This mark was used as the sediment was pushed up 2 mm above the top. This sediment was then scraped off into an enameled tray placed below the tube. The scraper and outer sides of the tube were rinsed with water, to ensure that all the sediment in the 2 mm section was retained. The sediment from the top 2 mm was then sieved through a 90 μm mesh sieve. No eggs or nymphs were lost from the sediment as the eggs measure approximately 300 μm x 200 μm (Hunt 1953).

After sieving, the sediment was placed in 20 mL scintillation vials and preserved with Kahle's fluid. Four drops of lignin pink dye in 95% ethanol were added to stain the eggs and nymphs so that they could be distinguished from the sediment. Later, the eggs

and first instar nymphs would be removed through sorting and flotation (Anderson 1959). These were kept in 70% ethanol.

The sediment in the bottom of the tube was also retained. It was rinsed through a 180 μm mesh sieve, placed in a jar and preserved with Kahle's fluid. Lignin pink dye was added to these samples. These can be later examined for *Hexagenia* eggs and nymphs, and other invertebrates. To ensure that the eggs collected were *Hexagenia* eggs, all the eggs collected from the cores were placed on slides and stained with CMC-9AF mounting medium. *H. limbata* and *H. rigida* were identified according to Koss (1968).

Results

General recovery characteristics

Hexagenia eggs stained a dark red colour with the lignin pink, making them highly visible in the sediment. The eggs could be distinguished from other red staining objects by a few factors. Under the dissecting microscope (25-50 x magnification) *Hexagenia* eggs were identified by their distinctive ovoid shape. The eggs also tended to stain a darker red colour than other objects in the sample that stained pink.

Most eggs retrieved from the cores were slide-mounted to verify the identification of *Hexagenia*. Under the compound microscope the eggs were confirmed to be *Hexagenia* eggs by the unique pattern of chorionic sculpturing. The chorionic sculpturing

consisted of large mesh reticulations formed either by straight ridges for *H. limbata*, or by strongly sinuous ridges for *H. ridgida* (Koss 1968).

Of the eggs collected (n = 1124), 88 % were checked, and of those 97 % were confirmed to be *Hexagenia* eggs. Of the *Hexagenia* eggs, 95.6 % were *H. limbata* and 4.4% were *H. rigida*. Organisms that were not *Hexagenia* eggs were removed from the results.

When I examined the eggs I classified them as being hollow, damaged, or entire. Hollow eggs were characterized by internal clarity and by having a longitudinal fissure extending about 3/4 of the way down the egg from which the nymph hatched (Hunt 1953). Damaged eggs were pieces of egg shell, eggs that broke apart when picked up or eggs that were soft. Entire eggs appeared to be undamaged and were firm. Entire eggs were assumed to be potentially viable.

Hexagenia nymphs were also recovered from the cores. They stained a light pink with the lignin pink making them harder to distinguish from the sediment. Recovery of the nymphs was done mainly through the flotation technique (Anderson 1959). The nymphs were classified as either newly-hatched (ca. 1 mm long , with relatively few segments on the cerci and antennae, and lack of pigmentation) or as previously-hatched (ca. 5-20 mm long, suggestive of at least 21 d growth under optimum conditions (Winter 1994)).

Table 3.2: The mean and standard deviation of entire, damaged, hollow, and total density of eggs found per m^2 (n=5) at the *Hexagenia*-present sites (sites 10 and 20) and the *Hexagenia*-absent sites (sites 12 and 25).

	Entire eggs/m^2		Damaged eggs/m^2		Hollow eggs/m^2		Total eggs/m^2	
Site	Mean	SD	Mean	SD	Mean	SD	Mean	SD
10	303	427.3	727	627.3	303	215.2	1333	460.6
20	1152	2081.8	182	269.7	303	524.2	1636	2830
12	182	269.7	61	136.4	182	269.7	424	506.1
25	242	454.5	303	427.3	303	427.3	697	796.9

Recovery of eggs and nymphs from reference cores

Hexagenia eggs were recovered from 60% or more of the reference cores (those to which eggs were not added) collected from the four sites in western Lake Erie. Damaged,, hollow and potentially viable eggs were found at all locations (Table 3.2). The two sites without *Hexagenia* nymphs (sites 12 and 25) had fewer eggs/m^2 found at the sites compared to the two *Hexagenia*-present sites (sites 10 and 20), but there was no statistically significant difference between the numbers of eggs collected at the sites (one-way ANOVA, $p>0.05$; see Table 3.3). Site 20 had the greatest number of eggs collected (1636 ± 2830 eggs/m^2) and the fewest eggs were collected at site 12 (424 ± 206.1 eggs/m^2). The total number of eggs per core found at each site are in Figure 3.2.

To summarize mean number of entire eggs per core at each site, the data were log transformed because one core out of 20 had 16 viable eggs, whereas the other cores had 0 to 3 eggs. The geometric mean number of potentially viable eggs per core ranged from 0.41 x/÷ 1.00 (site 25) to 1.52 x/÷ 2.20 (site 20) (Table 3.4).

Very few newly hatched nymphs were recovered from the cores; one nymph was recovered from a Site 10 core, and two from one Site 20 core.

Recovery of eggs and nymphs from egg supplemented cores

No corrections were made for naturally occurring eggs in the cores, since the numbers of apparently viable eggs and nymphs in the control cores were low and consistent at all four sites (Table 3.4).

Table 3.3: Summary of the one-way ANOVAs for the effects of the site on the presence of entire, hollow, damaged, and the total number of eggs (n = 5)

Dependent Variable	Variable	df	Mean square	F value
Entire eggs	site	3	11.294	0.82
	error	15	206.750	
Hollow eggs	site	3	0.196	0.13
	error	15	23.200	
Damaged eggs	site	3	4.582	2.53
	error	15	27.200	
Total eggs	site	3	15.462	0.57
	error	15	404.350	

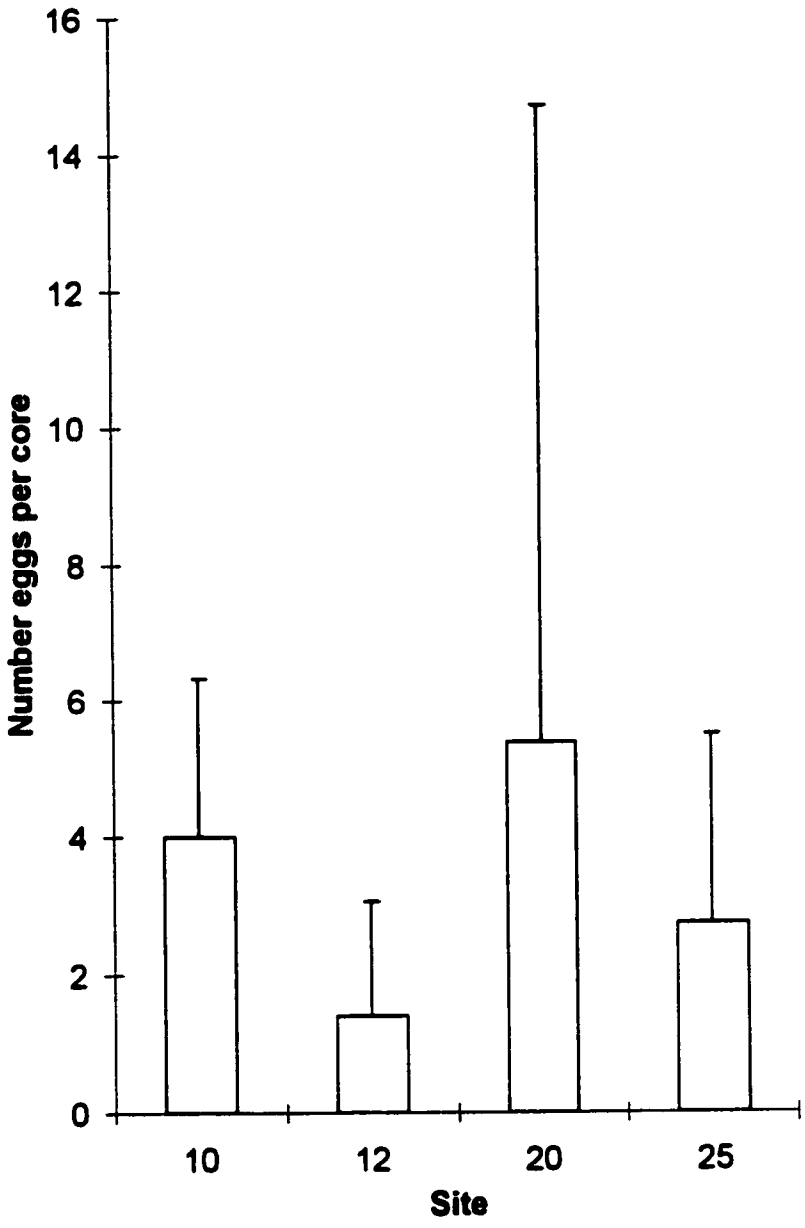


Figure 3.2: Mean number of total eggs (± SD) found in the control cores from each site. Site 10 and 20 are *Hexagenia*-present sites; sites 12 and 25 are *Hexagenia*-absent sites in western Lake Erie.

Table 3.4: Geometric mean number of potentially viable eggs per core (x/÷ SD) found at each of the 4 sites in the western basin of Lake Erie.

Site	*Hexagenia* populations present at the sites	mean eggs / core	x/÷ SD
10	yes	0.65	0.990
20	yes	1.52	2.201
12	no	0.43	0.667
25	no	0.41	1.00

A randomized block split-plot ANOVA (normoxic vs. hypoxic) was used to assess egg and newly hatched nymph recovery. The sites were blocked into sites with *Hexagenia* nymph populations present (sites 10 and 20) versus sites without nymph populations (sites 12 and 25). Each site was treated as a plot that was split into normoxic and hypoxic treatments.

Overall, site 10 had a low recovery of *Hexagenia* eggs and newly hatched nymphs. The cores collected from site 10 differed from the other cores in that they all contained 1-2 large (length ca. 1-2.5 cm), endemic nymphs. The nymphs were collected with the cores and were not removed throughout the experiment. The presence of the nymphs was evident from the burrows they made in the sediment and by their bioturbation which caused the water to be turbid.

Overall recovery of eggs

Overall recovery of eggs (hollow and entire) in the cores differed among the sites and the treatments. Site 10 had the lowest recovery rate in both the hypoxic and normoxic treatment cores (14%). Recovery of eggs in the normoxic treatments, for sites 12, 20 and 25 was lower than the recovery of eggs in the hypoxic core treatments (Figure 3.3). In the hypoxic treatments the recovery of eggs was 60 - 80%, whereas in the normoxic treatments the recovery rate ranged between 40 and 60%. The ANOVA showed that site ($p < 0.05$), the presence of *Hexagenia* at the site ($p < 0.05$) and the oxygen treatment ($p < 0.001$) all had a significant effect on the total number of eggs recovered (Table 3.5)

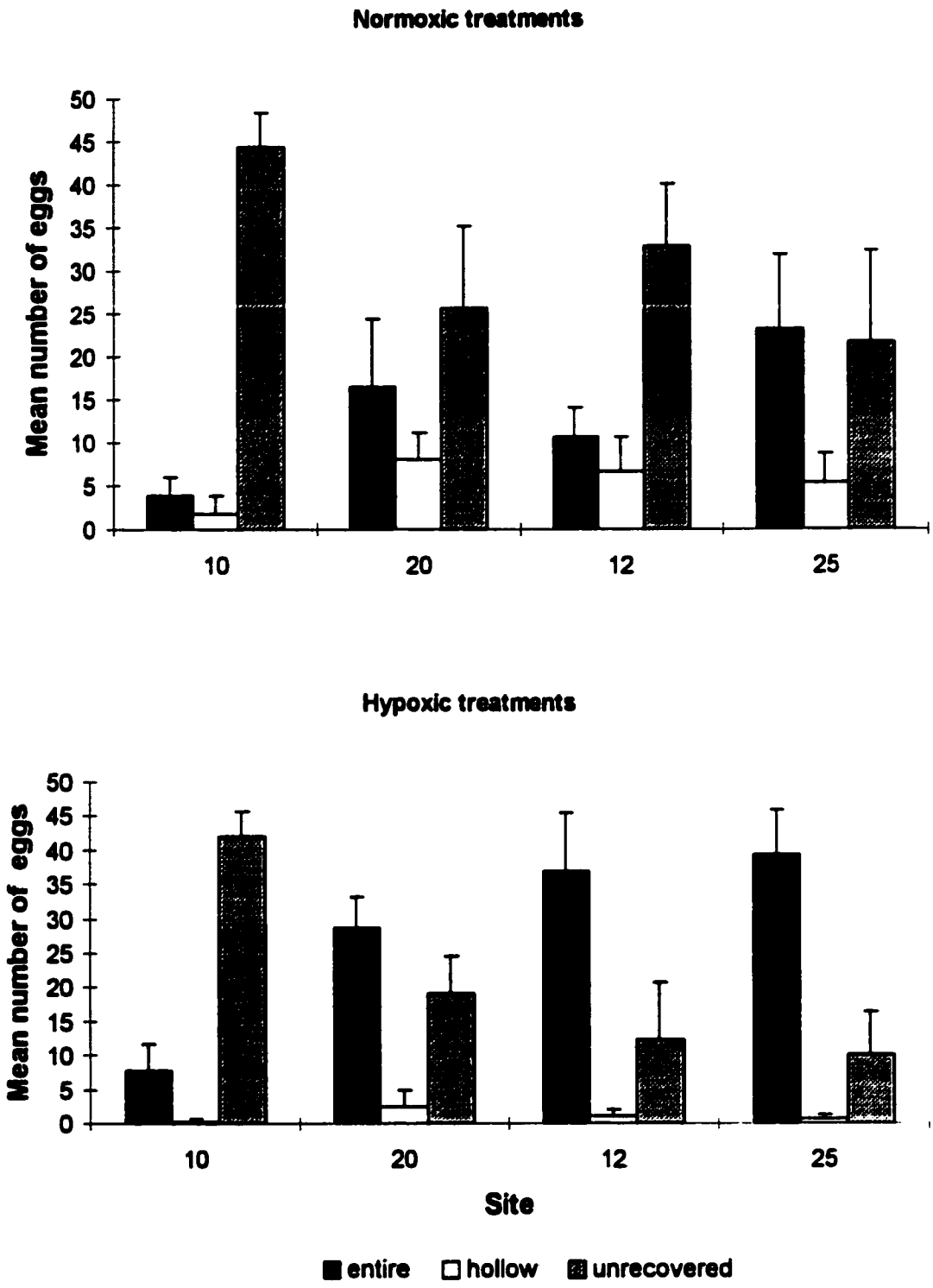


Figure 3.3: The mean number and standard deviation of planted *Hexagenia* eggs that were entire, hollow and unrecovered from the sediment cores (n=5), placed under normoxic or hypoxic conditions. Sites 10 and 20 are *Hexagenia*-present sites and sites 12 and 25 are *Hexagenia*-absent sites.

Table 3.5: Summary of the split-plot ANOVA for the effects of the presence of *Hexagenia* nymphs at the site, the site, the oxygen treatment, site x *Hexagenia* presence and site x treatment on the recovery of total number (entire + hollow) of *Hexagenia* eggs in the egg supplemented cores.

Variable	df	Mean Square	F-value
site	3	1425.133	11.55 *
Hexagenia nymphs at site	1	1988.100	16.11*
oxygen treatment	1	1020.100	8.27
site x treatment	3	123.367	99999.9 **
error	31	0.00	

* $p < 0.05$
** $p < 0.001$

Recovery of viable (entire) eggs

The split-plot ANOVA showed that the site and the treatment (normoxic vs. hypoxic) had a significant ($p < 0.05$) effect, and the treatment x site interaction had a very highly significant ($p < 0.0001$) effect on the recovery of entire eggs (Table 3.6).

The recovery of entire eggs, or unhatched eggs, was overall lower in the normoxic treatments compared to the hypoxic treatments. In the hypoxic treatments there were more

entire eggs recovered from the two sites without *Hexagenia* nymphs present at the sites than at the two sites with *Hexagenia*. Site 10, in both oxygen treatments, had a low recovery rate of entire eggs (4-8 eggs/core) compared to the other cores (10-40 eggs/core).

Recovery of hollow eggs

The split-plot ANOVA showed that the treatment (normoxic vs hypoxic) had a slightly significant ($p < 0.025$) effect, and the other factors, site, presence of *Hexagenia* at the site, and site x treatment interaction, had no effect on the incidence of hollow eggs (Table 3.7). There were more hollow eggs recovered from the normoxic treatment cores than the hypoxic treatment cores. Excluding site 10 with its low recovery rate, the number of hollow eggs (mean ± SD) collected from the normoxic treatments ranged between 6.6 ± 4.04 eggs/core (site 12) and 8.0 ± 3.16 eggs/core (site 20) (Figure 3.3 and Table 3.8). The hypoxic treatments had between 0.4 ± 0.89 hollow eggs/core (site 12) and 2.6±3.65 eggs/core (site 20) recovered.

Table 3.6: Summary of the split-plot ANOVA for the effects of the presence of *Hexagenia* nymphs at the site, the site, the oxygen treatment, site x *Hexagenia* presence and site x treatment on the recovery of entire *Hexagenia* eggs in the egg supplemented cores.

Variable	df	Mean Square	F-value
site	3	1156.967	6.37
Hexagenia nymphs at site	1	1904.400	10.48 *
oxygen treatment	1	2102.500	11.57 *
site x treatment	3	181.767	99999.9 **
error	31	0.00	

* $p < 0.05$
** $p < 0.001$

Table 3.7: Summary of the split-plot ANOVA for the effects of the presence of *Hexagenia* nymphs at the site, the site, the oxygen treatment, site x *Hexagenia* presence and site x treatment on the recovery of hollow *Hexagenia* eggs in the egg supplemented cores.

Variable	df	Mean Square	F-value
site	3	30.767	3.39
Hexagenia nymphs at site	1	0.900	0.015
oxygen treatment	1	193.600	21.35 *
site x treatment	3	9.067	1.41
error	31	6.435	

* $p < 0.025$

In terms of *Hexagenia*-present sites versus *Hexagenia*-absent sites there is no significant effect on the incidence of hollow eggs (Table 3.7). In the normoxic treatments there is no difference between the sites, except site 10 with it low recovery rate of eggs. In the hypoxic treatments site 20, a site where there are *Hexagenia* nymphs present had a slightly higher incidence of hollow eggs than the two sites without nymphs (sites 12 and 25).

Recovery of nymphs

The recovery of nymphs from the cores was relatively low (Table 3.8). Only one newly hatched nymph was recovered from site 10. The mean number of nymphs recovered from the site 20 cores was the same in both the normoxic and hypoxic treatment cores (3.6 nymphs/core). There were marked differences in the recovery of nymphs in the cores from the two *Hexagenia* absent sites. Oxygenated cores yielded means of 4.2 and 5.0 newly hatched nymphs (sites 12 and 25 respectively), whereas no nymphs were recovered from the hypoxic tubes.

The split-plot ANOVA showed that site, the presence of *Hexagenia* at the site, treatment, or the site x treatment interaction had no effect on the incidence of newly hatched nymphs (Table 3.9).

The number of nymphs recovered should be reflected in the number of hollow eggs as they both represent hatching. To determine the relationship between incidence of

Table 3.8: The mean (± SD) hollow eggs and newly hatched nymphs recovered from the egg supplemented cores for both the normoxic and hypoxic treatments. (n = 5)

	Hexagenia nymph	Normoxic cores		Hypoxic cores	
Site	population	Hollow eggs	New nymphs	Hollow eggs	New nymphs
10	yes	1.8 ± 2.05	0.2 ± 0.44	0.2 ± 0.45	0.0
20	yes	8.0 ± 3.16	3.6 ± 2.19	2.4 ± 2.61	3.6 ±6.07
12	no	6.6 ± 4.04	4.8 ± 4.44	1.0 ± 1.00	0.0
25	no	7.2 ±	5.8 ± 3.19	0.6 ± 0.55	0.0

Table 3.9: Summary of the split-plot ANOVA for the effects of the presence of *Hexagenia* nymphs at the site, the site, the oxygen treatment, site x *Hexagenia* presence and site x treatment on the recovery of the newly hatched *Hexagenia* nymphs in the egg supplemented cores.

Variable	df	Mean Square	F-value
site	3	22.967	1.00
Hexagenia nymphs at site	1	6.400	0.27
oxygen treatment	1	72.900	3.17
site x treatment	3	22.967	2.54
error	31	9.045	

hollow eggs and the incidence of newly hatched nymphs, linear regression was used to relate them in the 40 egg-supplemented tubes. Hollow eggs were found in 23 of these cores. In the cores where no hollow eggs were found there were also no nymphs recovered. The relationship between hollow egg and nymph recovery for the 23 cores was given by the regression equation:

$$\#\ \text{nymphs} = -0.25 + 0.75(\#\ \text{hollow eggs}) \qquad (3.1)$$

$t = 3.98$, $R^2 = 0.43$ (n=23). Spearman's rank correlation coefficient gave a value of $r_s = 0.75$ ($p < 0.001$). Equation 1 suggests that at least 75% of hollow eggs recovered were the result of hatching during the experiment. Up to 25% may have hatched previously.

Hatching

To determine the percent hatching in the cores it was assumed that the recovery rates of the entire and hollow eggs were equal. Based on the total (entire + hollow) number of eggs recovered, the percentage hatching was determined by the percent of hollow eggs recovered. To correct for the fact that not all the hollow eggs necessarily represented hatching from this experiment the percentage of hollow eggs was multiplied by 75% (Equation 3.1). The estimated percentage hatching for the sites and different treatments are summarized in Table 3.10.

The oxygen treatment had a highly significant ($p < 0.001$) effect on the percentage hatching, based on the split plot ANOVA (Table 3.11). Neither the site nor presence of

Hexagenia at the site affected the ability of the eggs to hatch. Hatching was greatest in the normoxic cores (14.2-26.8 %, sites 25 and 20 respectively) compared to the hypoxic treatment cores (1.2 %-5.8 %, sites 25 and 20 respectively) (Table 3.10).

By the end of the experiment 60% of *Hexagenia* eggs maintained in the petri dishes had hatched. Thus, the percentage of eggs estimated to have hatched in the cores was low by comparison.

Table 3.10: The mean ± SD percent hatching in the cores at the *Hexagenia*-present sites (sites 10 and 20) and the *Hexagenia*-absent sites (sites 12 and 25) in the normoxic and hypoxic treatments (estimated from hollow eggs and Equation 3.1).

	Percent hatching (mean ± SD)	
Site	normoxic	hypoxic
10	18.0 ± 17.43	3.0 ± 6.71
20	26.8 ± 10.57	5.5 ± 4.95
12	23.4 ± 13.43	2.1 ± 2.09
25	14.2 ± 5.68	1.2 ± 1.07

Table 3.11: Summary of the split-plot ANOVA for the effects of the site, the presence of *Hexagenia* nymphs at the site, , the oxygen treatment, and site x treatment on the percent hatching of *Hexagenia* nymphs in the egg supplemented cores (n=5).

Variable	df	Mean Square	F-value
site	3	127.689	2.82
Hexagenia nymphs at site	1	96.566	2.14
oxygen treatment	1	3117.774	68.98 **
site x treatment	3	45.201	0.52
error	31	87.656	

** $p < 0.001$

Discussion

General recovery of eggs

Hexagenia eggs were relatively easy to distinguish from the sediment and other organisms due to their ability to stain bright red with lignin pink and their distinctive ovoid shape and chorionic characteristics. *Hexagenia* eggs are naturally a translucent yellow-white colour. They also have an adhesive layer that surrounds eggs, allowing sediment to stick to the egg (Koss 1968). Both these factors make finding the eggs in the sediment difficult. Lignin pink stains the chitin in the shell of the eggs red (Cannon 1941) making the eggs visible in the sediment. Eggs were confirmed as *Hexagenia* by their chorionic sculpturing and ovoid shape (Koss 1968). Slide-mounting the eggs verified that 97% of the eggs recovered were *Hexagenia*, showing that eggs could be easily distinguished from other organisms.

The recovery of the nymphs was more difficult. When nymphs first hatch, they are translucent and the lignin pink only faintly stained them. The use of the flotation technique (Anderson 1959) after sorting through the sediment usually allowed the nymphs to be separated from the sediment.

When the eggs were retrieved, they were placed into one of three categories: entire, hollow, and damaged. Entire eggs, eggs that were not damaged in any evident way, and were firm when picked up, were assumed to be viable. Hollow eggs were empty shells with a longitudinal fissure that is formed when a nymph hatches out of the egg (Hunt 1953). Therefore, it was assumed that these eggs represented hatched eggs. The

third category comprised of pieces of egg shell, eggs that broke apart when picked up, or eggs that were very soft. I assumed that these eggs were in the sediment when the cores were collected. The *Hexagenia* eggs are very durable and should not have broken down within the time period of the experiment (pers. observ.).

Of the entire and hollow eggs recovered, it could not be ascertained which eggs were the added, newly oviposited eggs, and which were eggs that were in the sediment when the cores were collected. However, the reference cores consistently contained so few entire and hollow eggs, that any eggs collected with the sediment cores could be ignored (Table 3.2 and 3.4).

Recovery of eggs and nymphs from the reference cores

Hexagenia eggs were present at all four sites, both those with *Hexagenia* nymph populations (sites 10 and 20) and those lacking nymph populations (sites 12 and 25) in the western basin of Lake Erie. Entire, hollow, and damaged eggs were found in the reference cores collected from the sites (Table 3.2).

In terms of potentially viable eggs (entire eggs), there was no difference in the numbers of eggs found at the four sites (Figure 3.1). There was a mean (± SD, n=4) of 227 ± 367 eggs/m^2 found at each site.

The presence of entire eggs at the two *Hexagenia*-absent sites (12 and 25) means that potentially viable eggs are reaching these sites. The eggs could have been oviposited by gravid female imagoes, at the sites on the water's surface, or they may have drifted in through the water column from other areas. The *Hexagenia*-absent sites having the same

number of entire eggs as the *Hexagenia*-present sites may indicate that eggs are hatching at the *Hexagenia*-absent sites. If eggs were not hatching at these sites it would be expected that there would be more unhatched eggs found than at the *Hexagenia*-present sites.

The presence of hollow eggs, with the longitudinal fissure created when nymphs hatch out (Hunt 1953) further indicates that eggs are hatching in the *Hexagenia*-absent areas.

The presence of potentially viable eggs and hatched eggs, but not nymphs at the *Hexagenia*-absent sites suggests that some factor affects *Hexagenia* at the nymph stage of the life cycle. The nymphs are either dying at the sites, or they are emigrating once they hatch because conditions are not suitable for them.

Very few newly hatched nymphs were found in the reference tubes. Nymphs were only found at the *Hexagenia*-present sites. Since only 2 of the 20 reference cores produced newly hatched nymphs it can not be suggested that the eggs are not hatching at the *Hexagenia*-absent sites.

The newly hatched nymphs in the reference cores probably hatched during the experiment. The definition of the newly hatched nymphs was based on the size and lack of pigmentation. These nymphs in the reference cores would have come from eggs deposited the previous summer as the cores were collected before emergence and oviposition of eggs by adults (D. Laing and L.D. Corkum, University of Windsor, Windsor, ON, pers. comm.). The hatching of the nymphs would have been stimulated by the high temperature in the cores (22°C) compared to the field (12 °C, bottom temperature; pers. observ.)

(Flattum 1963, Friesen *et al.* 1979). This indicates that eggs can overwinter in the sediment and will hatch in the spring when the temperature rises above the hatching threshold of 8 -10 °C (Flattum 1963, Friesen *et al.* 1979, Giberson and Rosenberg 1992b, Chapter One). The ability of eggs to overwinter has been previously postulated, but not demonstrated (Heise *et al.* 1987, Giberson and Rosenberg 1992b).

Recovery of eggs and nymphs in the egg-supplemented cores

The original purpose of the egg-supplemented cores was to examine the effects of the sediment and anoxia on hatchability of the *Hexagenia* eggs at the four sites. Water column anoxia was not achieved in this study because the nitrogen gas used to deoxygenate the water ran out and I was unable to obtain more before the end of the experiment. Hypoxic conditions (O_2 < 6.0 mg /L) were created in the cores. Therefore, the effects of hypoxia were examined along with the sediment effects.

Before addressing the effects of the oxygen and site treatments on the hatching of the *Hexagenia* eggs, the overall retrieval rate of eggs from the cores will be discussed as the differences between sites have some interesting implications.

General recovery of eggs

I was unable to recover all the eggs that were placed into the sediment cores even with the use of lignin pink and flotation. Some of the unaccounted eggs may have been lost during the removal and sieving of the surface sediment. Transferring the top layer of sediment from the cores to the sieve, and from the sieve to the vials may have allowed for the loss of one or two eggs. Eggs would not have been lost through the 90 μm sieve used

as they measure 160-190 by 280-320 μm (Hunt 1953). Other eggs may have also remained in the cores if they were buried deeply in the sediment, or if they occurred in a depression in the sediment surface. The sediment surface was uneven and in some cases contained burrows and tunnels from organisms (*Hexagenia*, chrionomids, tubificids etc.) in the sediment. Another possibility is that eggs were missed during sorting, despite the staining.

There was a difference in my ability to retrieve the eggs from the cores among sites and treatments. Site 10 had the lowest overall recovery rate (14%) compared to the other sites (40% - 80%) (Figure 3.3)

The cores from site 10 were unusual in that each core contained 1-2 larger sized (1-2.5 cm) endemic nymphs that were collected with the sediment in the tubes. The nymphs burrow into the sediment and constantly beat their gills, creating a current through the burrow (Hunt 1953). This bioturbation mixes the top layers of sediment where the burrows are. It could account for the loss of eggs in the cores.

Only the top 2-5 mm of sediment was recovered and searched for eggs. *Hexagenia* burrows were 5-6 cm deep in the sediment, but they can go as deep as 12.5 cm (Hunt 1953). The eggs were possibly drawn down into the burrows, or they were buried deeper into the sediment, or eaten. The burying of the eggs by nymphs has important implications on the population dynamics of the nymphs.

Egg burial would either delay or prevent the hatching of the eggs. Hatching would be prevented when the eggs became buried in the anoxic layers of the sediment (Chapters One and Two; Fremling 1967). Anoxia would delay hatching until the eggs become re-

exposed to higher oxygen levels. The longer the eggs are buried in anoxic sediment the less likely they will hatch when exposed to normoxic conditions, as viability decreases with exposure to anoxia (Chapters One and Two; Fremling 1963). However, my observations of hatching in the reference cores verifies that at least some eggs remain viable over long term periods.

Eggs may not necessarily be buried down within anoxic layers. The aeration of the sediment caused by the burrowing of *Hexagenia* may still expose the eggs to oxygen, but at lower levels than the surface. The presence of oxygen in the sediment would allow the eggs to hatch, but not necessarily at the same time as eggs at the surface. Low levels of oxygen would delay the time to hatch by increasing the development time of the eggs as has been observed in copepods (Lutz *et al.* 1992), halibut (Helvik and Walther 1993) and lake herring (Brook and Colby 1980). The minimum oxygen required for the eggs to hatch is unknown. Hunt (1951) found that eggs buried 5-7.5 cm below stream silt were delayed in hatching by 2-3 weeks compared to unburied eggs.

The presence and bioturbation activity of large nymphs in an area could account for the multiple cohorts found in some populations (Heise *et al.* 1987). The nymphs could bury a certain percentage of the eggs, as in this study. If half the eggs became buried in the sediment where oxygen levels were lower than the surface, then two cohorts could be formed, provided all the buried eggs were exposed to the same oxygen level. The eggs at the surface, having a higher oxygen level, would hatch before the buried eggs. The difference in time to hatching between the surface group and the buried group would depend on the difference in the oxygen level. A 21 d delay in hatching between the two

groups would allow nymphs hatching from the sediment surface eggs to grow 5-20 mm larger than nymphs emerging from the buried eggs, under optimum conditions.(Winter 1994).

The actual percentage of eggs that became buried in the sediment would depend on the density of the nymphs; the higher the density the greater the number of eggs that would become buried. If all the eggs became buried, then only one cohort would be found in the area of the nymphs. This could still lead to different cohorts being formed between areas where there are high densities nymphs versus areas of very low densities.

This may be a unique density-dependent mechanism, whereby the size structure of a population is regulated by physical activities of conspecifics, rather then by competition for resources.

Treatment also had an effect on the recovery of the total numbers of eggs (hollow and entire). More eggs were recovered in the hypoxic treatment cores than in the oxic treatment cores. This may reflect the fact that more eggs hatched in the oxic cores. It is possible that the recovery rate of the hollow eggs and nymphs was less than the rate for entire eggs.

Hatching

Hexagenia nymphs and hollow eggs were recovered from oxygenated cores from both the *Hexagenia*-present sites (sites 10 and 20) and the *Hexagenia*-absent sites (sites 12 and 25) (Table 3.7). This indicates nymphs can hatch in sediments from all sites under normoxic conditions.

The percentage of eggs hatching among the four sites under the normoxic conditions did not vary significantly (Table 3.8). I assumed that if there was a difference in the ability to hatch among the sites in the oxygenated cores, the difference would be due to sediment contaminants. The presence of at least some sediment contaminants can prevent or delay the hatching of the *Hexagenia* (Friesen 1979). Since the percentage hatching did not significantly differ among the sites, any sediment contaminants present at any of these sites were not at levels sufficient to affect the percentage hatching any more than at the other site.

Even though hatching occurred in the normoxic tubes, the percentage hatching of the eggs in the cores was relatively low (18-29 %) compared to the hatching observed in the control eggs in the petri dishes without sediment (60%). The lower hatching rate in the cores could be either due to the presence of the sediment slowing the rate of development or reducing the viability. The sediment biological oxygen demand could reduce the oxygen concentration at the sediment-water interface, and therefore slow egg development.

The oxygen treatments did have an effect on the hatching of the *Hexagenia* eggs. The recovery of entire eggs ($p < 0.05$), hollow eggs ($p < 0.025$), and the percentage hatching ($p << 0.001$) were all significantly affected by the oxygen treatments (Tables 3.6, 3.7, and 3.11). Overall more eggs hatched in the normoxic treatments than in the hypoxic treatments (Table 3.9 and 3.10, Figure 3.3).

The lower levels of oxygen in the hypoxic cores probably reduced the rate of development of *Hexagenia* eggs in the tubes, therefore reducing the number of hatched

eggs collected from the hypoxic cores. The hypoxic conditions could have also increased the mortality of the embryos, compared to the eggs in the normoxic cores. Because the eggs removed from the hypoxic and normoxic treatment tubes were immediately preserved, I could not determine if unhatched eggs were viable. Therefore, I could not determine if the reduced hatching in the hypoxic cores was due to delayed development, increased mortality, or a combination of both. Hypoxic conditions can increase the incubation time, and has reduced the survivorship of embryos of copepods (Lutz *et al.* 1992), and in fishes (Brooke and Colby 1980, Helvik and Walther 1993).

It would be useful to determine the effects of decreasing oxygen concentrations on *Hexagenia* embryo survivorship and development. From the hypoxic treatment cores it can be determined that oxygen concentration between 3.0-6.5 mg/L do not prevent the development of the embryos since nymphs hatched, but the development rate was reduced compared to the higher oxygen levels in the normoxic cores (8.0 mg/L). The minimum oxygen concentration required for *Hexagenia* development and hatching is unknown. Chapters One and Two indicated that anoxia (O_2 < 1.0 mg/L) completely arrests the development of *Hexagenia* eggs and reduces the survivorship.

Conclusion

Overall, the results from this study indicate that the absence of nymph populations south of Middle Sister Island (site 25) and in the middle of the Pigeon Bay area, west of Point Pelee (site 12) is not due to the lack of eggs reaching the sites. The reference cores contained as many eggs at the *Hexagenia*-present sites as at the *Hexagenia*-absent sites.

Since the eggs were able to hatch under normoxic conditions in the sediment from all four sites examined, contaminants in the sediments are probably not inhibiting the hatching of the eggs. It is still possible that the nymphs could be affected by the sediment at the *Hexagenia*-absent sites. The third factor examined in this study was the effect of low epibenthic oxygen levels. Hypoxia significantly inhibited the hatching of the eggs. Hypoxia slows the development rate of the eggs, delaying the time to hatching (Brook and Colby 1980, Lutz *et al.* 1992, Helvik and Walther 1993). It is known from Chapters One and Two that anoxia actually inhibits the hatching of the *Hexagenia* eggs. Based on the above factors it seems that the presence of eggs and sediment contamination effects on the hatching of eggs are not the controlling factors in preventing the recovery of *Hexagenia* populations in some regions of western Lake Erie.

It is unknown if there are episodes of anoxia actually occurring in the area south of Middle Sister Is. and in the Pigeon Bay region where there is an absence of *Hexagenia*. It would be useful to have continuous oxygen meters placed in these areas to record the oxygen levels and determine if anoxic events are taking place. Daily monitoring of anoxia is needed at these sites because anoxia in the western basin is intermittent and would last 1- 4 days if it occurred (Bartish 1984). One or more events of anoxia per year in these areas would prevent the establishment of nymphs as the nymphs will die within 48 h of exposure to <1.0 mg O_2 /L (Hunt 1953) even with eggs present in these areas.

From this study, the burial of eggs by nymph bioturbation was also observed. Burial of eggs by the nymphs and subsequent resurfacing, and hatching of the eggs at

different times could lead to the multiple size frequencies found in some *Hexagenia* populations (Heise *et al.* 1987).

GENERAL CONCLUSIONS

My studies have shown that *Hexagenia* mayfly embryos will not develop or hatch under anoxic conditions. The *Hexagenia* embryos can survive periods of anoxia and will hatch, with reduced success, once re-exposed to oxygen.

The development of *Hexagenia* embryos was inhibited when the eggs were placed into an anoxic environment. This was indicated by the time to hatching of the eggs being the same, and under some conditions increased, in the incubation conditions of 20 °C in normoxic water, after being in anoxic conditions. If development was occurring in the anoxic conditions then the time to hatching when placed in normoxic, 20 °C water would be reduced after being in anoxia.

Temperature did have an effect on the development of the *Hexagenia* embryos under anoxic conditions. Eggs that were held at 4 °C in anoxic conditions for 28 d, required 2-4 d longer to hatch at room temperature then those maintained at temperatures > 8 °C in anoxic condtions. This effect of a delay in hatching at incubation temperatures was also observed in eggs after they were held in the normoxic conditions at 4°C.

The longer the *Hexagenia* embryos were held in anoxic conditions the lower the viability became. Viability decreased as a log function of the time in anoxia. After 8 mo in anoxia at 20 °C the hatching success of the embryos was 16 % (35 % of the viability in normoxic conditions at 20 °C).

I also studied the effects of temperature on the development of *Hexagenia* under normoxic conditions. My results concurred with those found in the literature, where the

development rate decreased with a decrease in temperature (Flattum 1963, Friesen 1967, Friesen *et al.* 1979, Wright *et al.* 1982 and Giberson and Rosenberg 1992). Development was arrested at temperatures below 11 °C. The 4 °C temperature, as stated above, actually caused an increase in development time when the eggs were brought up to the incubation conditions of 20 °C.

Initially, I studied the effects of anoxia on eggs in the early stages of development (1-2 d old). The effects of anoxia on mid (7 d old) and late (12 d old) developmental stage embryos were also examined. Again, development was delayed while the eggs were in anoxic conditions. The delay in hatching was equal to the time the eggs were in the anoxic conditions.

In terms of viability, the late stage eggs had the highest viability. After being in anoxia for 28 d, the percentage hatching dropped approximately 5 % (from approx. 88% to 83%). The viability of the mid stage embryos decreased by 18 % after 28 days in anoxic conditions (from approx. 85 % to 67 %) and was therefore affected the most by the anoxic conditions. The viability of the early eggs stayed the same after 28 days in anoxia at 77 %. The viability in the previous experiments where early stage eggs were used to examine the effects of anoxia at different temperatures did show a decrease in viability similar to the mid stage eggs. These results indicate that embryos can be exposed to anoxia at any stage of their development and survive.

I also attempted to examine the effects of anoxia on the eggs in sediment by placing *Hexagenia* eggs in cores containing sediment from western Lake Erie. Due to problems with making the cores anoxic, hypoxic conditions were examined instead. The

sediment was taken from four sites in the basin, two with established *Hexagenia* populations and two without.

The number of hatched eggs in the cores indicated that differences in the sediment among the four sites had no effect on the hatching. If toxic contaminants were present at the sites, especially those without nymphs it then did not inhibit the hatching of the eggs. Hypoxia did delay the hatching in the cores. Fewer eggs hatched under the hypoxic conditions than in the normoxic conditions.

Cores were also collected from the four sites to determine if there were *Hexagenia* eggs present at these sites. Eggs, both potentially viable and hatched were found at all four sites. There were no significant differences among the sites. This indicates that eggs are reaching those sites without *Hexagenia* nymphs and the eggs could be hatching.

I concluded, from the above results, that eggs were reaching the two areas lacking *Hexagenia* nymph populations in western Lake Erie and that the sediment was not inhibiting the hatching of the eggs, but low oxygen concentrations would delay the hatching. Since eggs are reaching the areas and can hatch, then some factor may be affecting the nymphs and preventing them from becoming established in some areas of the western basin..

Noteworthy observations of nymph bioturbation effects on the eggs were made from the cores collected at one site containing 1-2 endemic nymphs that were 1.5-2 cm long. The recovery rate of the eggs that were placed in these cores for the experiment was very low. I speculate that the eggs became buried deeply in the sediment (below the 2 mm removed from the surface to recover the eggs) by the burrowing action of the

endemic nymphs. This was only a side observation to my project but it would be important to further explore the implications of egg burial by the nymphs.

The fact that *Hexagenia* eggs can tolerate anoxic conditions can be important to the nymph population, especially in a system such as the western basin of Lake Erie. The western basin has experienced short term (1-4 d) anoxic events from transient stratification (Britt 1955a, Carr *et al.* 1965, Bartish 1984). *Hexagenia* nymphs are sensitive to anoxic conditions and were eradicated by anoxia in the western basin in the summer of 1953 (Britt 1955a). The population was able to recover the following year (Britt 1955b). One possible reason for the recovery the following year was that there were eggs present at the time that survived the anoxic event and hatched when normoxic conditions returned.

Another way that eggs can be exposed to anoxia is by becoming buried in the sediment. Burial of the eggs and later re-emergence to oxic conditions and hatching could lead to the multiple cohorts found in some *Hexagenia* populations (Heise *et al.* 1987). Benthic samples from western Lake Erie suggest that populations in the basin are in transition from a single cohort to multiple cohorts (Ciborowski *et al.* 1997) It is possible that some cohorts can be linked to major storm events that cause a batch of eggs to resurface from the sediments and hatch.

It would be useful to determine the extent of an egg bank of *Hexagenia* eggs in western Lake Erie. Knowledge of the presence of eggs in the sediment, how deeply they

are buried and how viable the buried eggs are would give more of an indication of the importance of buried eggs to the *Hexagenia* population.

Since there are several of areas in the basin where *Hexagenia* have not reappeared and one of the factors that may be preventing the establishment of nymphs is intermittent periods of anoxia it would be useful to monitor the epibenthic oxygen concentrations. Continuous monitoring of the oxygen levels would determine if anoxic events that would kill the nymphs were occurring in those areas preventing populations of nymphs from occurring there.

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APPENDIX I: Pilot study of the effects of anoxia and temperature on *Hexagenia* embryos

Initially, the study on the effects of anoxia at different temperatures on *Hexagenia* embryos (Chapter One) was carried out in the summer of 1996. This first study determined problems in the proposed methodology and gave some preliminary results.

Methods

Egg Collection

Hexagenia eggs were collected at night, from the Lakeview Marina Lighthouse on the Detroit River in Windsor, Ontario, on August 13, 1996. Three replicates of eggs from 4 female imagoes were collected and placed in Whirl-pak bags filled with aerated dechlorinated water. The females laid their eggs on the surface of the water. The females were later removed and placed in 70 % ethanol. The eggs were placed in treatment the following day.

Experimental design

The experimental design was a 2 (oxygen) x 4 (temperature) x 4 (days in treatment) factorial design with 3 replicates. *Hexagenia* eggs were placed in 20 mL scintillation vials containing declorinated water, that was either at anoxic or normoxic

oxygen levels. The vials containing the eggs were placed in incubators set at at 4°C, 8°C, 16°C or 22°C for 0, 1, 4, 7, and 28 days. After treatment, eggs were placed into hatching conditions in aerated dechlorinated water at 22 °C. Here, they were monitored daily for hatching (See Chapter One).

Treatments

While the eggs were in treatment they were stored in 20 mL scintillation vials. Initially, all the treatment vials for a replicate were each filled with 100 eggs that were counted out and placed into a vial with about 2 mL of dechlorinated aerated water. The vials were then randomly assigned to one of the treatments.

For the anoxic treatments, anoxic water was produced by bubbling 99.95% nitrogen gas through an air stone into 1000 mL of dechlorinated water for at least 30 minutes. Once all the eggs were counted out and placed into the scintillation vials the anoxic water was pipetted into the vials while blowing nitrogen gas over the surface of the vial. Immediately after filling the vial to the top with the anoxic water, the vial was capped while continuously blowing nitrogen gas over the surface of the vial (A.H. Warner, University of Windsor, pers. communication). The vial was then sealed with Parafilm.

Treatment vials for the normoxic treatments were filled with aerated dechlorinated water, capped and sealed with Parafilm.

Once a set of vials for one replication was filled and sealed the vials were placed in environmental chambers set at one of the four treatment temperatures 4 °C, 8 °C, 16 °C or 22 °C. After all the replicates were placed in treatment, vials were removed from each

oxygen and temperature treatment at 0 (approx. 3 hrs after being placed in treatment) , 1, 4, 7 and 28 days and placed into hatching conditions.

Hatching conditions

To place the eggs into hatching conditions, a set of vials was removed from each treatment temperature and placed at 22 °C for 1 h. This allowed the water and eggs to acclimate to 22 °C. The vials were uncapped and most of the treatment water was carefully removed, without taking any eggs, using a pipette. Some water was left behind with the eggs. The eggs were then rinsed into 60 mm diameter x 15 mm deep petri plates with aerated declorinated water.

The eggs were then monitored daily, under a dissecting microscope, for hatching. All hatched nymphs were counted and removed during the monitoring. Monitoring continued until it was presumed that no further hatching would occur as indicated by the eggs browning or deteriorating internally (Friesen *et al.* 1979). Time to first hatch, time to 50% hatch and percent viability were determined from the daily hatching.

Statistical analysis

An analysis of variance (SAS Institude Inc. 1985) was performed to determine the effects of the replication, time in treatment, treatment temperature, treatment oxygen level and all their interactions on the time to hatching and viability of the *Hexagenia* embryos.

Results and Discussion

Problems with the methodology and recommendations

The first problem encountered was counting out the *Hexagenia* eggs for each vial. The eggs were very sticky and adhered to each other, the petri dishes (both glass and plastic), and the forceps and Pasteur pipettes used to pick up the eggs to transfer them to the vials. This made the process of counting out the eggs very time consuming and difficult. Clay could be added to the eggs to prevent the sticking (J.J.H. Ciborowski, University of Windsor, pers. communication). Another option is to add an approximately equal volume of eggs to each vial. This would reduce the time and manpower needed to count the eggs for each vial. In this study the number of eggs per vial (mean ± S.D, n=60) was 97.7 ± 11.2 when recounts were made at the end of the study.

There were also problems with the environmental chambers maintaining their temperature during the experiment as later determined by temperature loggers that were placed in each incubator. The 4 °C incubator constantly had its temperature ranging from -4 °C to 12 °C daily, with an average temperature of 5 °C. The 8 °C incubator had a temperature ranging from 10 to 12 °C for the first 5 d of the experiment; it then rose to 23 °C and remained there for the rest of the experiment. The 16 °C chamber was at 20 to 21 °C for the whole experiment. The 22 °C chamber was relatively constant at 22 °C throughout the experiment. Because of this, treatment temperatures used in the analysis below were adjusted to the approximate average temperature of the incubators (5, 11, 20.5 and 22 °C).

Another problem encountered in the methodology was that eggs started to hatch in the 28 d treatment vials at 20.5 and 22 °C , before removal from treatment. It was unknown when hatching started in those vials, but hatching was noted on day 24 of the experiment.

It is expected that *Hexagenia* eggs would hatch around day 14 of the experiment at 22 °C with oxygen present, as that is the normal hatching time at that temperature (Flattum 1963, Hanes 1992). Because of this an extra removal day from treatment should be included in the design at day 14. This would be the last removal day for the 22 °C normoxic treatment. All the other treatments would still have eggs in treatment until day 28.

Hatching not only occurred in all the normoxic treatment vials, but also in most of the anoxic vials at 20.5 and 22 °C . Problems with the probe of the Clark Style O_2 microelectrode (Diamond General Inc, Ann Arbor, MI) that was to be used to measure the oxygen prevented me from measuring oxygen concentrations during the pilot study.

Since hatching occurred in all but one anoxic vial, it was presumed that the water in the anoxic treatments was not truely anoxic. This was either due to oxygen getting into the vials before being sealed, and not being consumed by the eggs, or there was leakage of oxygen into the vials throughout the experiment. The first senerio seems most probable since initially there was 2 mL of aerated water in the vials that was never removed, because it would mean removing eggs. Also, there was an air bubble at the top of the vials in the vials where hatching occurred.

To reduce the amount of oxygen in the anoxic vials, initially, it was recommended that the eggs should have the anoxic water filtered off and then be placed in anoxic water before being placed in treatment (A.H. Warner, University of Windsor, pers. communication). Also, when the vials are filled, they should be filled to the top, so that there are no air bubbles in the vials.

Viability and development

Since anoxic conditions were not confirmed in the anoxic treatments, and hatching occurred in many of the anoxic treatments (indicating oxygen was present) oxygen treatments will be referred to as low and high oxygen treatments.

Only the data for the day 0, 1, 4, and 7 were examined for the effects of low and high oxygen levels at different temperatures on development and viability of *Hexagenia* embryos. This is because as indicated above, hatching occurred in the 28 day treatment vials before removal of the eggs from treatment.

No eggs at the 11 °C treatment for 7 days were included in the results either, because the temperature rose to 23 °C on day 5 of the experiment.

A four-way analysis of variance was done on the data to test the effects of the replication, treatment temperature (5, 11, 20.5 and 22 °C), oxygen level (0 = low, 1 = high), days in treatment (0,1,4, and 7d) and their interactions on time to hatching and viability.

Development

Development was affected by the temperature and the time in treatment, but not by the oxygen levels (Figure I.1). This indicated that there was enough oxygen in the low oxygen treatments for development. The effects of temperature were as expected from the literature, in that the longer the eggs were at the colder temperatures, the longer the time to hatching (Flattum 1963, Fremling 1967, Friesen *et al.* 1979, Wright *et al.* 1882, Giberson and Rosenberg 1992b).

Time to first hatch at 22 °C was 15 d, and time to 50% of total hatching was 17 d, as determined by the day 0 treatments. The eggs at 11°C and 5 °C for 4 and 7 d had a delay in hatching equal to the time in treatment. i.e. the time to hatch when under hatching conditions was equal to the normal incubation time at 22 °C. This indicates that no development was occurring at these temperatures. Friesen *et al.* 1979, determined the lower threshold temperature for development of *Hexagenia* eggs to be between 8 and 12 °C.

The ANOVAs for the effects of replication, treatment temperature, treatment oxygen, days in treatment and their interactions on time to hatching are shown in Table I.1 and I.2. Both time to first hatching (Table I.1) and time to 50% of total hatching (Table I.2) were examined. Time to 50% of total hatching (median of viable eggs) was used instead of time to 50% hatching of the total number of eggs (median of eggs in treatment) because very few of the day 4 treatments had over 50% hatching success.

The ANOVAs indicated that only days in treatment and the interaction between temperature and days in treatment had a very significant effect ($p < 0.0001$) on the time to both first hatching (Table I.1) and time the 50% of total hatching (Table I.2). As indicated

Table I.1: Summary of the four-way ANOVA of the effects of replication, treatment temperature, treatment oxygen, days in treatment and their interactions on time to first hatching of *Hexagenia* embryos.

Variable	df	Mean square	F value
replication (rep)	2	1.375	1.48
treatment temperature (temp)	1	0.341	0.37
temp x rep	2	0.153	0.16
days in treatment (day)	1	325.692	349.66***
day x rep	2	0.293	0.31
temp x day	1	163.586	175.62***
temp x day x rep	2	0.183	0.20
treatment oxygen (oxy)	1	0.336	0.36
rep x oxy	2	0.356	0.38
temp x oxy	1	2.397	2.57
temp x rep x oxy	2	0.610	0.65
day x oxy	1	.691	0.74
day x rep x oxy	2	1.092	1.17
temp x day x oxy	1	1.990	2.14
temp x day x rep x oxy	2	1.684	1.81

** : $p < 0.0001$

Table I.2: Summary of the four-way ANOVA of the effects of replication, treatment temperature, treatment oxygen, days in treatment and their interactions on time to 50% of total hatching of *Hexagenia* embryos.

Variable	df	Mean square	F value
replication (rep)	2	0.627	0.73
treatment temperature (temp)	1	2.050	2.37
temp x rep	2	0.237	0.27
days in treatment (day)	1	339.855	393.43***
day x rep	2	0.254	0.29
temp x day	1	167.735	194.18***
temp x day x rep	2	1.301	1.51
treatment oxygen (oxy)	1	0.065	0.07
rep x oxy	2	0.0786	0.09
temp x oxy	1	0.463	.54
temp x rep x oxy	2	0.0433	0.05
day x oxy	1	0.084	0.10
day x rep x oxy	2	1.172	1.36
temp x day x oxy	1	0.377	0.04
temp x day x rep x oxy	2	1.114	1.29

** : $p < 0.0001$

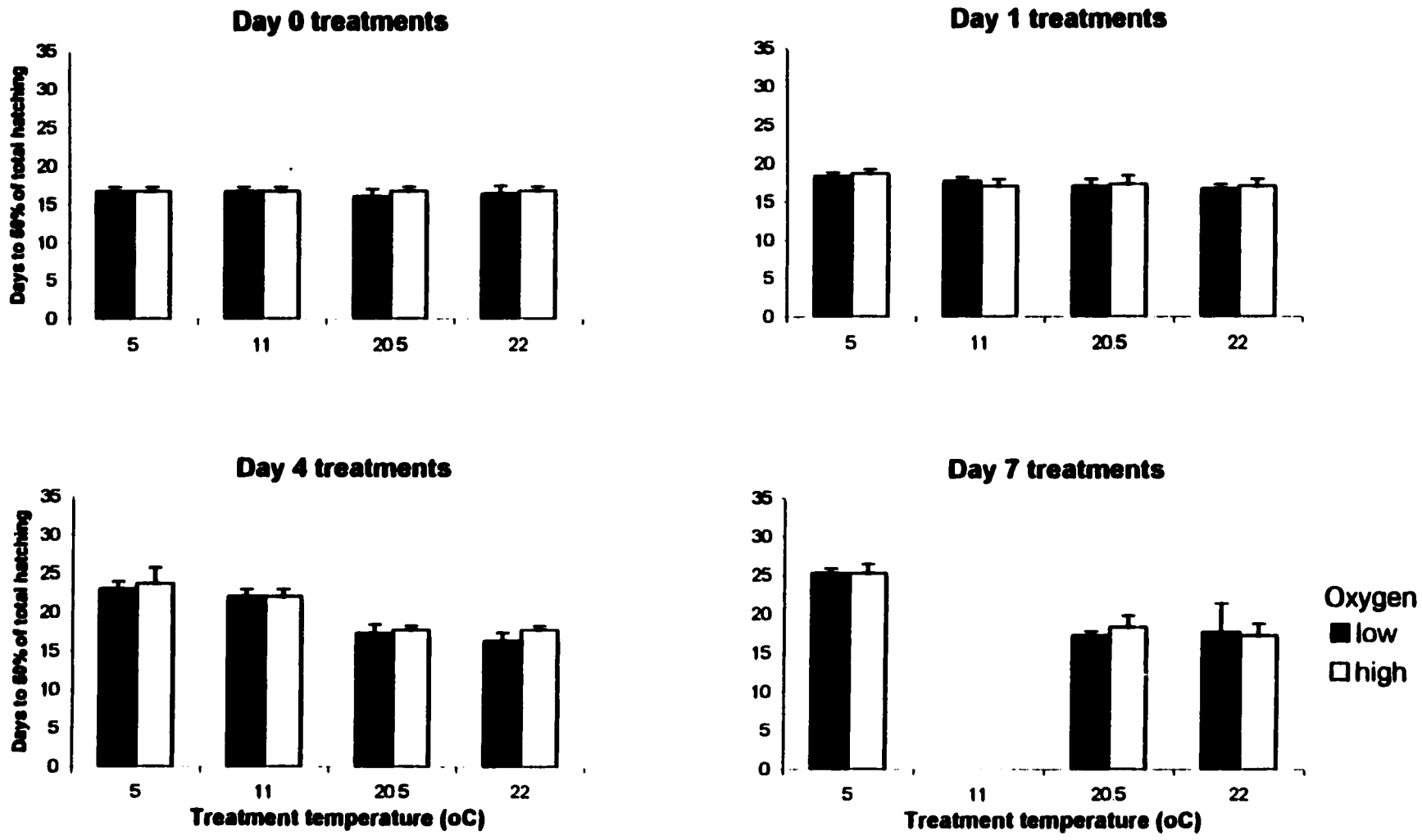

Figure I.1: Time to 50% hatching of total hatching (mean ± S.D.) of *Hexagenia* embryos after being in high and low oxygen treatments at temperatures of 5, 11, 20.5 and 22 °C for 0, 1, 4, or 7 d (n=3)

above the longer the *Hexagenia* embryos were held at the lower temperatures the longer they took to hatch.

Differences between the hypoxic oxygen and normoxic oxygen treatment effects on viability were only evident after 7 days in treatments (Figure AI.2). Generally, at the high temperatures (20.5 and 22°C) treatments the low oxygen treatments had a higher viability

Viability

Viability (mean ± SD %) of the *Hexagenia* embryos was initially high at 81.0 ± 9.7 % hatching in the day 0 treatments.

The effects of the treatments on viability on the eggs can be seen in Figure I.2. The day 4 treatments had a much lower overall viability than all the other treatments. This was probably due to a difference in the source of water being used for the eggs when they were placed into the hatching conditions.

In the hypoxic oxygen treatments temperature had an effect, in that the 5 °C treatments had a lower viability than the higher temperature treatments. There was no significant difference in the viability among the different temperature treatments in the high oxygen treatments.

The ANOVA on the effects of the variables on viability showed that only the number of days in treatment had a very significant effect ($p < 0.001$) (Table AI.3). The replication, treatment temperature, treatment oxygen level and all the interaction terms had no significant effect on the viability.

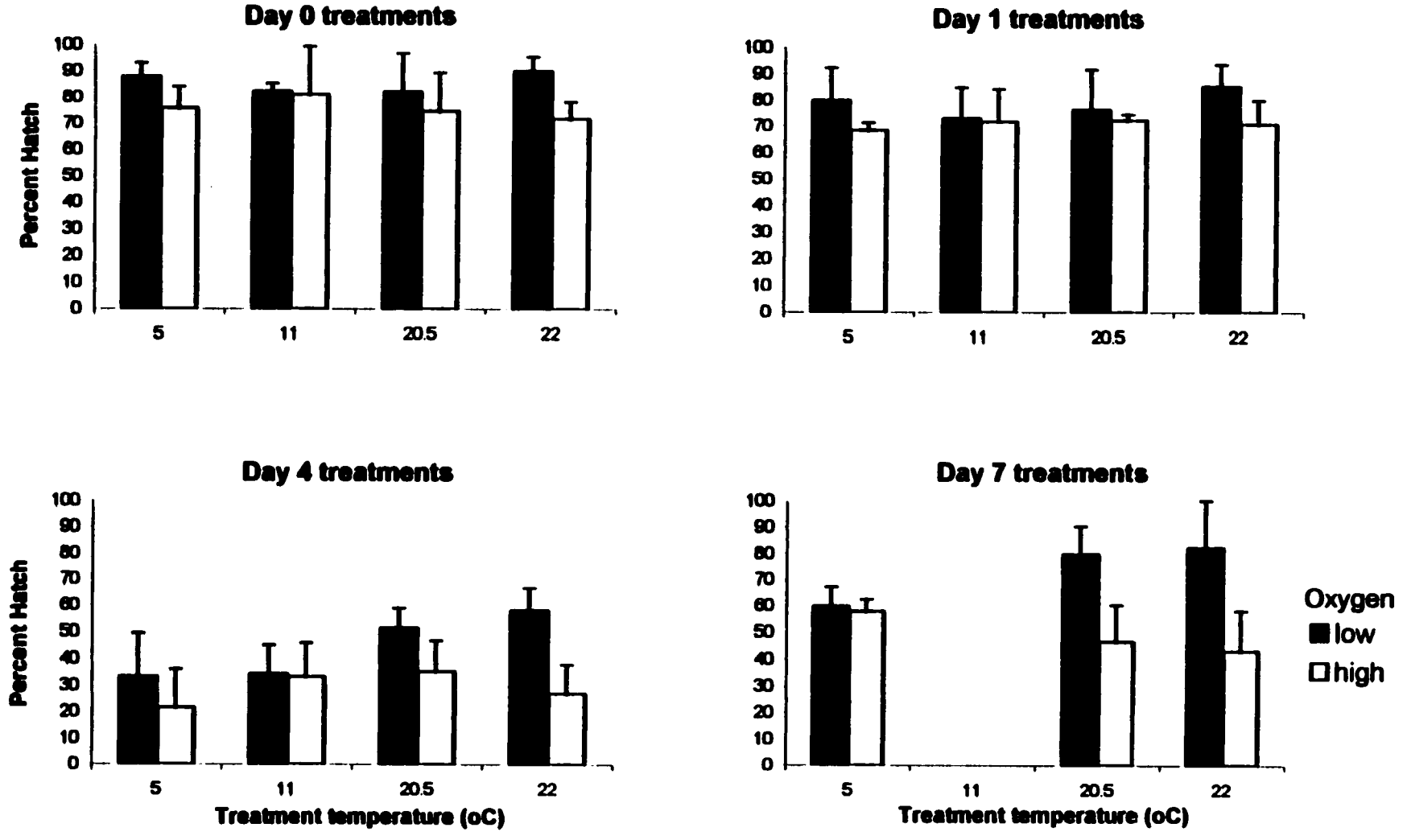

Figure I.2 Time to 50% hatching of total hatching (mean ± SD) of *Hexagenia* embryos after being in high and low oxygen treatments at temperatures

Table I. 3: Summary of the four-way ANOVA of the effects of replication, treatment temperature, treatment oxygen, days in treatment and their interactions on the viability of *Hexagenia* embryos.

Variable	df	Mean square	F value
replication (rep)	2	185.969	0.54
treatment temperature (temp)	1	3.129	0.01
temp x rep	2	6.617	0.02
days in treatment (day)	1	4658.601	13.46**
day x rep	2	358.786	1.04
temp x day	1	472.837	1.37
temp x day x rep	2	187.777	0.54
treatment oxygen (oxy)	1	150.042	0.43
rep x oxy	2	17.093	0.05
temp x oxy	1	0.152	0.00
temp x rep x oxy	2	41.153	0.12
day x oxy	1	132.901	0.38
day x rep x oxy	2	14.034	0.04
temp x day x oxy	1	695.743	2.01
temp x day x rep x oxy	2	2.621	0.01

**: $p < 0.001$

The decrease in viability over time in the high oxygen treatments was thought to be due to oxygen consumption of the developing eggs reducing the oxygen level. The lower oxygen levels later on in development could affect the eggs more than a constantly low oxygen level as in the low oxygen treatments. The viability in the low oxygen treatments was higher than the high oxygen treatments, yet the time to hatching of the eggs was the same. This indicates that either the eggs were developing under the low oxygen conditions, or the rate of development increased when brought to the hatching conditions at 22 °C.

If the oxygen levels in the high oxygen treatments are being reduced significantly by oxygen consumption then the water will have to be changed regularly in the vials to maintain high oxygen levels (> 6.0 mg/L). An oxygen consumption study should be done on the eggs (Appendix II).

Long-term effects of anoxia

The one vial at 22°C in the low oxygen, 28 day, treatment that had no hatching in it was actually kept sealed for 2.5 months. It was then opened, the eggs were placed in aerated dechlorinated water at room temperature and monitored for hatching. Time to first hatching was 9 d after being placed at hatching conditions, time to 50% of total hatching was 10 d and the viability was 72 %.

It was assumed that the water in that vial was anoxic and *Hexagenia* eggs could survive long term exposure to anoxia. *Hexagenia* eggs have been shown to survive anoxia for 110 d (Fremling 1967). The reduction in time to hatching was probably to initial development at the beginning of treatment when there was still oxygen present in the vials.

Conclusions

i) Too much oxygen was getting into the anoxic treatments, therefore the procedure of placing the *Hexagenia* eggs into anoxia has to be modified.

ii) The viability of *Hexagenia* eggs was greater in the high oxygen treatment than the low oxygen treatments, after being in treatment for 7 days.

iii) *Hexagenia* eggs can survive anoxic conditions for up to 2.5 months.

APPENDIX II: Oxygen consumption in the treatment vials and by *Hexagenia* eggs

Introduction

The viability of *Hexagenia* eggs in the normoxic treatments in Appendix I and Chapter One decreased the longer the eggs were sealed in the treatment vials. Initially, when this happened, I was concerned that the oxygen concentration in the vials was being reduced, by oxygen consumption of the eggs over time and this was reducing the hatching success.

In the study described in Appendix I, I did not measure the oxygen concentrations at the end of the treatments and therefore, I did not know how much the oxygen concentration decreased in the vials. The initial oxygen concentration of the water placed in the vials was assumed to be ~ 8.5 mg /L O_2 (normal atmospheric saturation at 20 °C) as the water had been aerated. To have normoxic conditions the oxygen concentration was to be maintained at levels >6.5mg /L water.

When the study was repeated in 1997 (Chapter One), I decided to replace the water in the vials every 4 d to re-introduce oxygen into the vials. Oxygen measurements taken when vials were opened after treatment indicated that oxygen levels in the vials were decreasing to levels below 6.5 mg/L in some cases.

I decided, that before repeating the experiment in 1998, to do a pilot study to determine how long it took for the oxygen concentration in the vials to decrease below 6.5 mg/L. Based on this, I could establish how frequently to change the water in the vials to

maintain an oxygen concentration above 6.5 mg /L water (60% of normal atmospheric saturation at 20 °C). At the same time I hoped to determine the oxygen consumption rate of *Hexagenia* eggs.

Methods

Egg collection

The *Hexagenia* eggs were collected on 15 June 1998, at Colchester Harbour, Colchester, Ontario, Lake Erie. The eggs were collected from individual female imagoes as in Chapter 1 and 2. One replicate consisted of eggs collected from one female. The eggs were placed in aerated, dechlorinated water in Whirl-pak bags.

Experimental procedure

The oxygen consumption of the *Hexagenia* eggs at 22 °C was to be determined over a five day period. For each replicate (n=5), 5 vials were filled with aerated dechlorinated water to which 100-300 *Hexagenia* eggs/vial were added. Blank vials (n=3/day) containing aerated water, but no eggs, were also filled and sealed at the same time. The blanks were used to determine additional oxygen losses, besides that from the eggs, in the vials. The vials were capped and sealed with Parafilm wax.

Not enough water was initially aerated to fill all the replicate vials at the same time. Replicates A and B were first filled with one set of blank vials, then replicates C-E were filled, along with two sets of blank vials.

Over the next 5 consecutive days, after the vials were filled, one vial per replicate and three blank vials were removed, opened and the oxygen concentration of water was measured. The oxygen level was measured with a Clark Style O_2 Microelectrode (Diamond General Inc., Ann Arbor, MI).

Results and Discussion

Oxygen levels in the treatment vials

Oxygen concentrations were quite variable among the vials, whether they were blank, or whether they contained eggs (Table II.1). However, oxygen concentrations in all the vials on day 5 were lower than initially, suggesting that significant respiration occurred in both blanks and in the vials containing the eggs.

After 5 d, the measured oxygen concentrations in the vials for replicates (rep) A and B, and blank (blk)1 were lower than in rep C-E, and blks 2 and 3 (Table II.1). At the end of the experiment, the oxygen concentration was 5.1-5.5 mg /L in rep A and B, and ranged from 5.8-6.6 mg /L in rep C-E.

The difference in the oxygen consumption in between the two sets of vials (Rep A & B vs. Rep C-E) may be accounted for by the fact that two different batches of water, that were aerated at different times, were used to fill the vials. Rep A and B, and blk 1 vials were filled with one batch of aerated water, and the rest of the vials (rep C-E, blk 2-3) were filled with a second batch of water.

The initial oxygen concentration of the water placed in the vials was not measured, but was assumed to be 8.6 mg /L water, normal atmospheric saturation at 22 °C . From the blanks it can be seen that the oxygen concentration was higher in blk 2-3 than in blk 1 (Table II.1). If there was a difference in temperature between the two batches of water the initial oxygen concentration of the water would have differed, because the solubility of oxygen in the water changes with temperature(Wetzel 1975). A 1 °C difference in temperature, which did occur in the chambers regularly (pers. observ.), from 21 °C to 20°C, would change the normal atmospheric oxygen saturation level of the water from 8.68 mg /L to 8.84 mg /L (Wetzel 1975).

The decline in the oxygen concentration in all three blanks (Table II.1) also suggests that biological activity (respiration) other than eggs contributed to oxygen consumption, possibly bacterial contamination. It is possible that the first batch of water was more contaminated, and therefore had a greater oxygen consumption rate than in the second batch. This would also account for the lower oxygen levels in Rep A, B and Blk 1 compared to Rep C-E, Blk 2 and 3.

Concentrations in two of the five replicates had declined to 6.5 mg/L (the lower boundary of normoxia) within 3 d. Therefore, I decided to replace the water in the vials for the 1998 study (Chapter One) every two days. This would ensure that the oxygen levels would not reach levels below 6.5 mg/L in any of the normoxic treatment vials.

Table II. 1: The oxygen concentrations (mg/L) measurements, over 5 days, in the vials containing *Hexagenia* eggs (replications A-E) and in the blank vials (Blank 1-3) without eggs.

	Oxygen concentrations (mg/L) in the vials							
Day	Rep A	Rep B	Rep C	Rep D	Rep E	Blank 1	Blank 3	Blank 3
0	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6*
1	7.3	7.2	7.5	7.7	7.9	8.4	8.6	8.6
2	6.7	6.3	8.0	7.9	7.9	8.1	8.3	8.3
3	6.5	6.2	7.2	7.6	7.4	7.6	8.1	8.1
4	5.5	5.8	5.9	6.0	6.6	6.8	7.8	7.7
5	5.1	5.5	6.3	5.8	6.6	6.3	8.0	7.3

* oxygen levels on day 0 were assumed to be at normal atmospheric saturation levels at 20°C .

Oxygen consumption of the eggs

The cumulative oxygen consumption was initially calculated for the different replicate vials. To calculated the cumulative oxygen consumption in the vials on each day the measured oxygen concentration in a vial was subtracted from the assumed oxygen concentration on day 0 (8.6 mg/L). Since there was oxygen being consumed in the blank vials, and I wanted to determine the oxygen being consumed by the eggs, the oxygen used in the blank vials was subtracted from oxygen consumed in the replicate vials. Blk 1 was subtracted from rep A and B, and the average of blks 2 and 3 were subtracted from rep C-E, to account for the two batches of water used. Based on the above calculation cumulative oxygen consumption decreased between day 2 and 5 in rep A and B when the cumulative oxygen consumption should have been increasing. Therefore, rep A-B were not used to determine the oxygen consumption of the eggs.

Replicates C-E were used to determine the oxygen consumption of the eggs. The cumulative oxygen consumption per egg for a replicate $vial_i$ was calculated by Equation 1. The oxygen consumption in Equation 1 refers to the cumulative oxygen consumed in the vial.

$$\text{Cumulative oxygen consumption/ egg} = \frac{((O_2 \text{ consumption/ replicate } vial_i) - (O_2 \text{ consumption/ blank}))}{(\text{number of eggs} / vial_i)} \quad (1)$$

The cumulative oxygen consumption per egg over the 5 d of the study is graphed in Figure II.1. A regression equation was fitted to the data using the STATISTICA© software

program. The relationship of the cumulative oxygen consumption over time is given by the equation:

$$\text{Cumulative oxygen consumption} = -0.233 + 1.983\ (\text{day}) \qquad (2)$$

The regression analysis of equation 2 is given in Table II.2.

The oxygen consumption of the eggs / hr was also calculated. The average values of the replicates (C-E) for the different removal days is depicted in Figure II.2. The oxygen consumption µg/L / egg / hr for day 3-5 of the experiment were similar with an average (± SD) of 0.077 ± 0.021 µg/L / egg / hr. The oxygen consumption on day 1 is higher than the other days, while on day 2 it is lower.

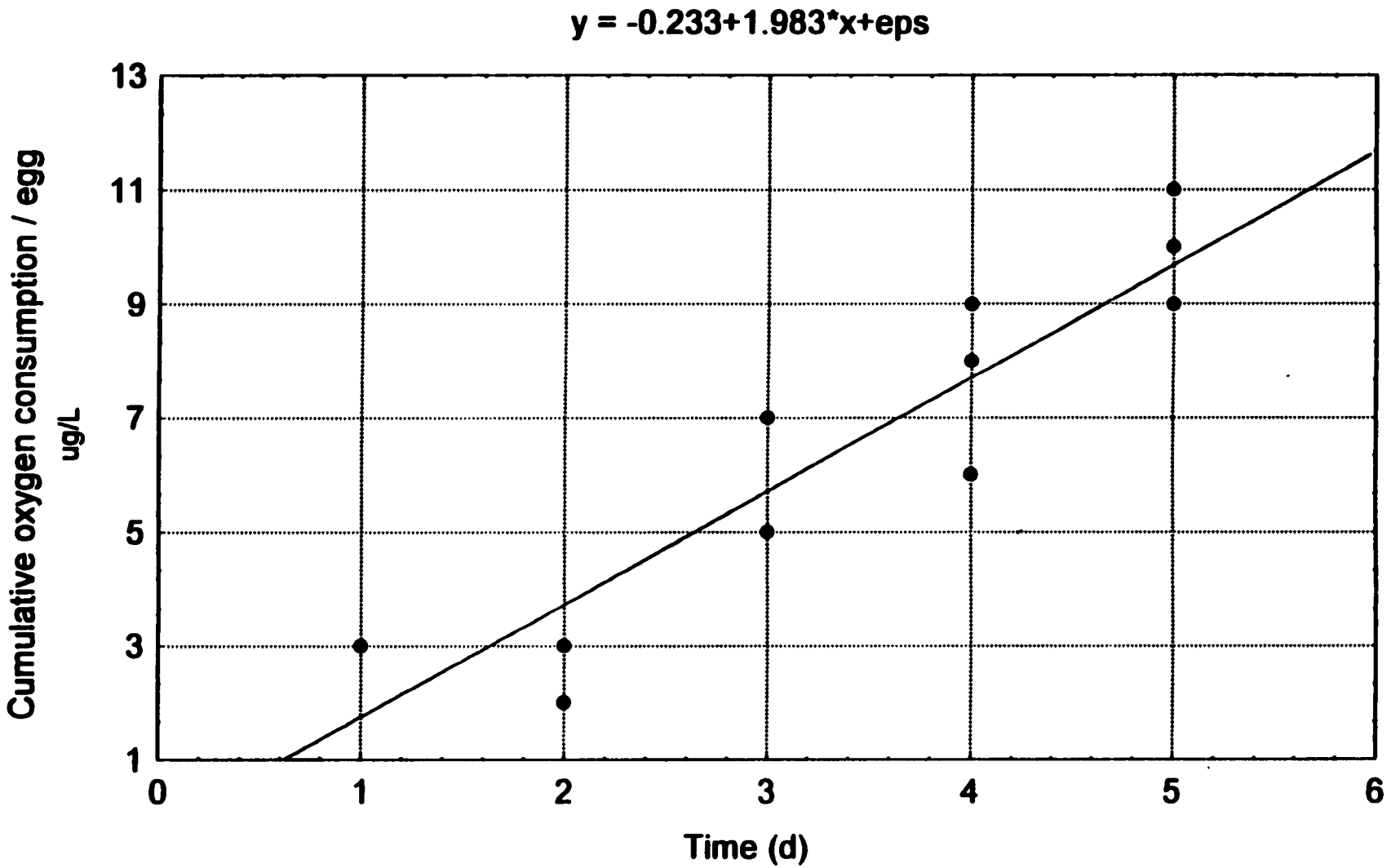

Figure II.1: Cumulative oxygen consumption µg/L/egg of *Hexagenia* eggs.

Table II.2: Regression analysis of equation 2.

Model fitting results				
Independent Variable	Coefficient	Est. std. error	t	R^2
Intercept	-0.233	0.775		
time (d)	1.983	0.234	8.49 ***	0.89

*** $p < 0.001$

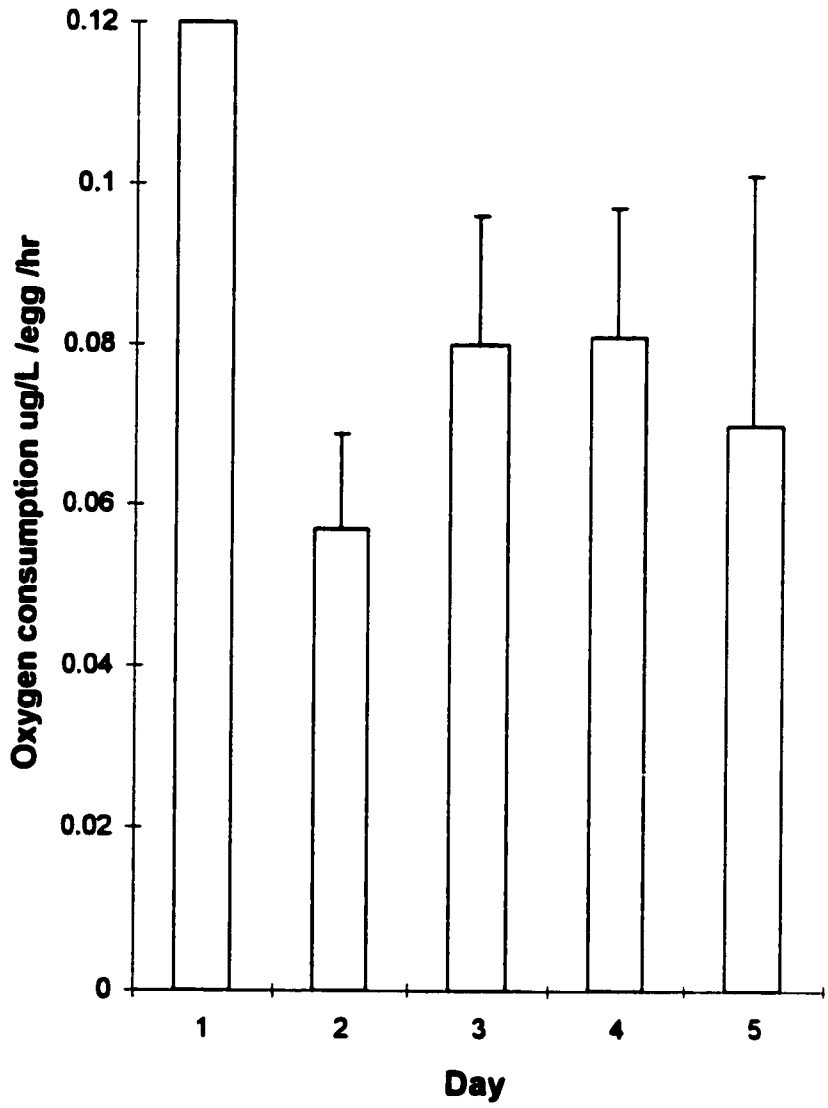

Figure II.2: The oxygen consumption (ug/L/egg/hr) of *Hexagenia* embryos.

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